Avian Diseases

FEBRUARY 1958

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Avian Diseases

VOL. II: No. 1, February 1958

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PUBLISHED FOUR TIMES A YEAR BY THE CORNELL VETERINARIAN, INC., ITHACA, NEW YORK

For subscriptions in the U. S., Canada, and territories and possessions thereof, address the Business Manager; price $5.00 a year. For all other foreign subscriptions $7.00 per year. In sterling area mail to Bailliere, Tindall and Cox, 7-8 Henrietta St., Covent Garden, London, W. C. 2, England. Subscription rates are subject to change without notice.

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AVIAN INFECTIOUS HEPATITIS.† I. CLINICAL AND PATHOLOGICAL MANIFESTATIONS*

By M. SEVOIAN, R. W. WINTERFIELD and C. L. GOLDMAN

Department of Veterinary Science
University of Massachusetts
Amherst, Massachusetts

Received 9 July 1957

DURING recent years, a primary liver degeneration in chickens of flock significance has been observed in the Poultry Disease Diagnostic Laboratories of Massachusetts. Until October 1956 all attempts to isolate an infective agent at this station had failed.[7] To date numerous isolations from five poultry flocks have been made here in embryonated chicken eggs and the disease has been reproduced experimentally. Many field cases where isolation attempts failed were diagnosed presumptively from histological sections.

Several descriptions of primary liver degenerations are found in the literature. Marthedal[4] has described degenerative foci in the liver of predominantly young chickens. He stated that these lesions were grossly similar to those of blackhead. Comprehensive bacteriological examinations, aerobic and anaerobic, were negative; so were repeated inoculations into embryonated chicken eggs. Lukas[3] reported the occurrence of a nonsuppurative hepatitis without hemorrhage or necrosis in chickens caused by a pleomorphic, coccoid, gram-negative organism. Tudor[6] described a liver degeneration with hemorrhage and necrosis in chickens in New Jersey occurring over a period of twenty years. A causal agent was not isolated.

* Contribution No. 1113 of the Massachusetts Agricultural Experiment Station.

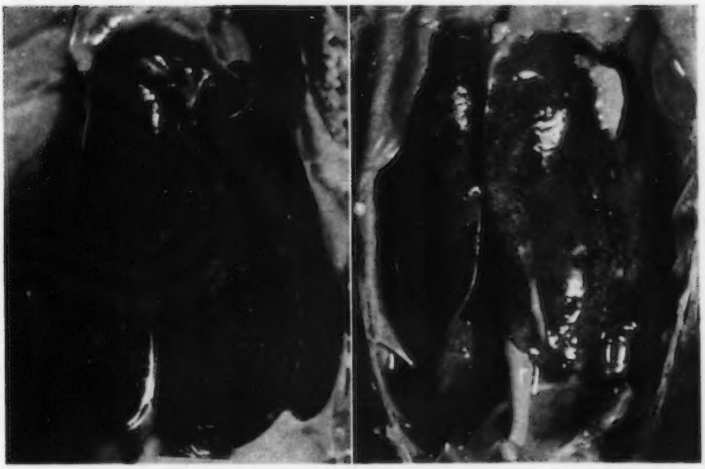
† Presented before the general session, ninety-fourth annual meeting, American Veterinary Medical Association, Cleveland, Ohio, Aug. 19-22, 1957.

Delaplane *et al.*[2] reported the isolation of an agent causing a primary hepatitis with focal degeneration and eosinophilic infiltration. In chicken embryo assays, the agent was resistant to aureomycin, terramycin, and penicillin but was adversely affected by streptomycin.

SIGNS AND LESIONS IN NATURAL INFECTIONS

The signs of avian infectious hepatitis in chickens under field conditions in Massachusetts usually had an insidious onset. More often, though not always, a small percentage (up to 10 percent) of the birds in a given flock appeared affected at any one time. Egg production lagged where laying pullet flocks were involved, or decreased as much as 35 percent during variable lengths of time with older flocks. Usually the disease lingered in an untreated flock for many weeks. Severely affected birds were listless and roosted or stood apart from the rest of the flock. They died very suddenly when forced to exercise. Chickens less affected were active but their combs usually were shrunken, shriveled, dry and scaly. Except acutely affected individuals, infected birds tended to "go light". In flocks severely involved, mortality ranged up to 10 or 15 percent during the course of several weeks. To date, the disease has been diagnosed in chickens 10 weeks and older from flocks in Massachusetts. Both light and heavy breeds have been affected.

Gross lesions. On necropsy the most striking features of the disease in older chickens under field conditions were degenerative and hemorrhagic changes in the liver. There was variability in the severity and extent of lesions from bird to bird or even from lobe to lobe within a given liver. Some of the livers were slightly enlarged and mahogany-brown but otherwise had no gross lesions. Other livers had pin-head size, grayish-white, necrotic foci often along the lobar edges. More severely affected livers were firm and friable and had irregularly round (asterisk or cauliflower-like) necrotic areas up to one centimeter in diameter distributed throughout the organ (figures 1, 2). On cut surface the necrotic foci appeared interconnected by cords of degenerative cells. Occa-



Figs. 1 (left) and 2 (right) Photographs of livers with focal degeneration from mature chickens naturally infected with avian infectious hepatitis.

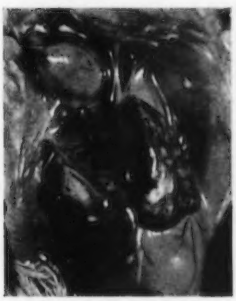


Fig. 3. Photograph of an 18-day-old chicken embryo inoculated with the infectious hepatitis agent (9539) in the amniollantoic cavity at 10 days of incubation. Note the focal changes in the liver and the paleness of the heart.

Fig. 4. Photograph of the organs of an experimentally infected 9-day-old chicken with focal degeneration in the liver and inflammation in the heart. The bird was necropsied 7 days post inoculation.

sionally there were irregular areas of hemorrhage and red "bubble-like" lesions (hematocysts) under the liver capsule. Sometimes severe hemorrhage ruptured the capsule releasing blood into the body cavity. Ascites and hydropericardium were seen; hearts and kidneys were enlarged and pale. A catarrhal enteritis was found in a small percentage of affected birds.

In younger chickens (10 weeks of age) the hearts were more severely and consistently involved than in mature birds. The heart muscle appeared pale and flaccid. A straw-colored gelatinous exudate clung to the epicardium whereas a more watery material filled and sometimes distended the pericardial sac. Gross liver lesions were less evident in semimature chickens than in laying hens.

Microscopic lesions. In mild or early infections, lymphocytic foci sometimes containing a few eosinophilic granulocytes were seen microscopically most often around the portal areas of the liver. Fatty metamorphosis, congestion, vascular dilation, and hemorrhage commonly were observed in the liver. In more severe cases, lymphocytic and granulocytic foci and focal degeneration were predominant changes (figure 5). The areas of degeneration which were irregular in size and shape did not bear consistent relationship to lobular structure. An increase of connective tissue was seen around the portal and interlobular areas. Proliferation of bile duct epithelium and the formation of additional bile ducts were common. In an occasional liver, there were granulomatous areas with fibrinoid necrosis. Sometimes the liver capsle was edematous and infiltrated by inflammatory cells (figure 6). In the advanced stages of the disease, the spleen had decreased lymphopoietic activity (figure 7) and occasionally had granulomatous lesions. The kidneys had focal areas of heterophils and lymphocytes, often in interstitial areas; the parenchymal cells in some focal areas were degenerated and these areas were infiltrated by inflammatory cells, predominantly heterophils. Dilated tubules and tubular casts containing heterophils were not uncommon findings (figure 8). The bone marrow of affected chickens had increased numbers of large immature

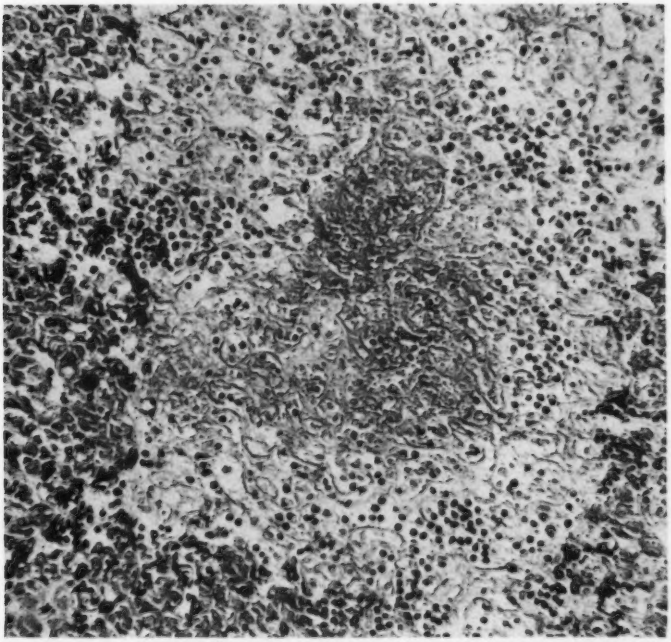


Fig. 5. Photomicrograph of a liver section with focal degeneration and granulocytic infiltration from a naturally infected mature chicken (240×).

myelocytes. In young chickens mononuclear cell infiltration of the epicardium and myocardium was more common and pronounced than in mature birds.

ISOLATIONS FROM NATURAL INFECTIONS

Livers from suspected flocks routinely were cultured for aerobic and anaerobic bacterial pathogens at the time of necropsy. At the same time, livers were seared and then stabbed with sterile cotton swabs. Each of the swabs was placed in 1.5 ml of sterile saline. In later isolations, however, livers or hearts, or both, were minced, suspended in saline with antibiotics, and centrifuged.[8] Whenever possible, saline suspensions or supernatants were inoculated within the same day

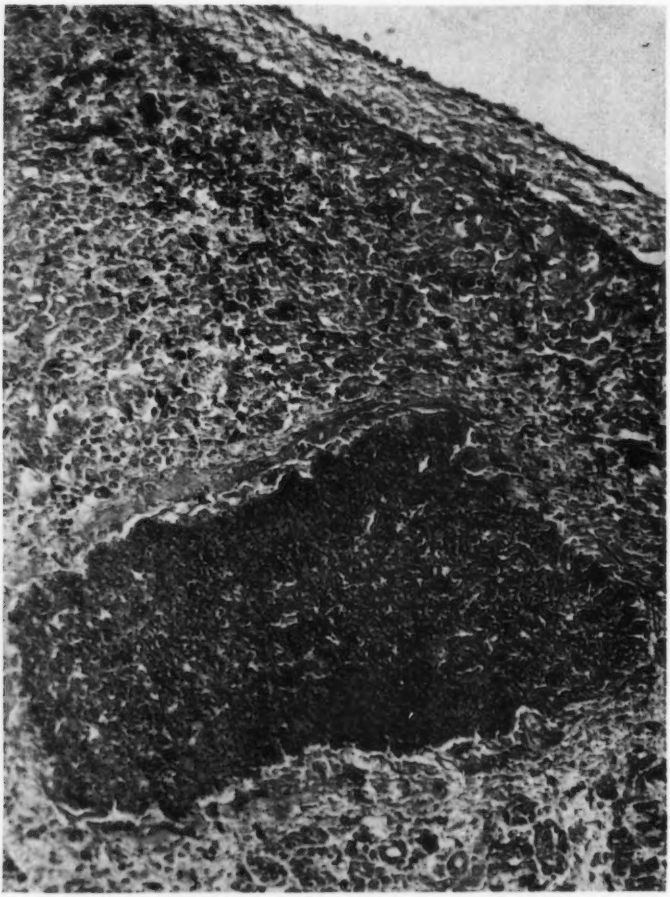


Fig. 6 Photomicrograph of a liver section with a granulomatous lesion, thickened capsule and subcapsular hemorrhage from a naturally infected mature chicken (204×).

into six-day-old embryonated chicken eggs via the yolk sac using 0.1 ml of the inoculum per egg. Where positive isolations were made, embryos died usually on the 4th and 5th days post inoculation and had cutaneous and subcutaneous congestion and hemorrhage. Harvested yolk material from

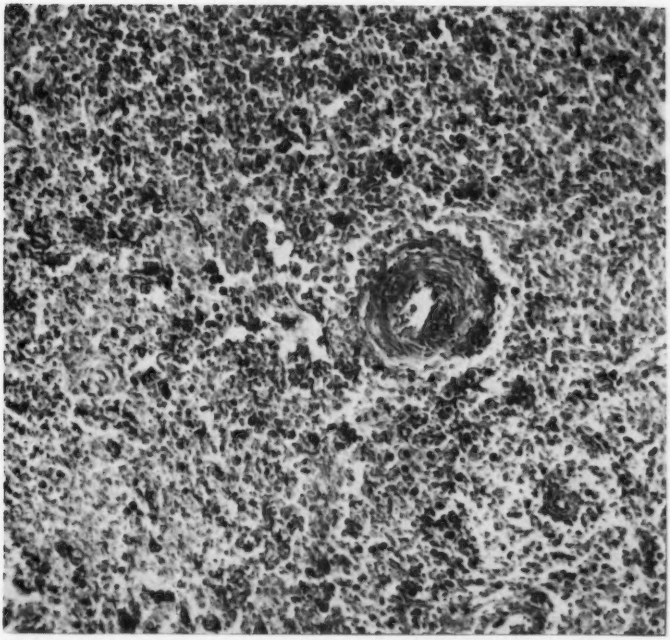


Fig. 7. Photomicrograph of a spleen section with decreased numbers of lymphoid cells from naturally infected mature chicken (240×).

dead embryo yielded no growth on blood and chicken infusion agar and in thioglycolate medium. PPLO broth and agar (Difco) cultures also were negative. When the agent subsequently was propagated in the allantoic cavity of ten-day-old chicken embryos, deaths generally were delayed and occurred from 3 to 11 days post inoculation. Embryos dying in the last trimester of incubation had enlarged, pale spleens and greatly enlarged livers with congestion and focal degeneration (figure 3). Such pathologic changes were of considerable value in tentatively identifying the agent prior to chicken inoculation.

EXPERIMENTAL PROCEDURES

Tissues and blood from 80 Rhode Island Red chicks from two separate trials were examined. Half of the chicks in

each trial was inoculated with the infectious hepatitis agent (IHA, 1297) harvested in the form of yolk fluids of infected chicken embryos (2nd passage), and the other half was maintained as control birds. The stock, derived from a closed flock known to be free of chronic respiratory disease, was reared in isolation. They were fed a commercial ration. At 2 days of age the birds were given 0.1 ml of the agent intraperitoneally. Hemagglutination inhibition, serum neutralization, and rapid serum plate tests for Newcastle disease, infectious bronchitis, and chronic respiratory disease respectively were negative throughout the trials.

Blood and tissues were saved from two chicks from each of the infected and control groups on alternate days (3 times weekly) up to 3½ weeks post inoculation. Blood was withdrawn from the heart with a syringe and needle and placed in tubes containing balanced oxalates. Blood smears were stained with Wright's stain for differential counts. Erythrocyte and leukocyte counts were made according to the technique outlined by Natt and Herrick.[5] Direct readings were taken from a Spencer hemoglobinometer. The rest of the blood examinations were made according to standard procedures outlined by Coffin.[1] Tissues were fixed in Bouin's fixative and cut six microns thick. They were stained with hematoxylin and eosin.

The effect of the IHA on older birds was investigated. The effect of the agent on 3½-week-old chickens already has been reported.[7] Fifteen laying female and two male 18-month-old Rhode Island Red chickens were given 0.25 ml of the IHA intraperitoneally. An equal number of control birds was maintained. The birds were kept for 9 weeks and then sacrificed and examined for lesions. Isolation attempts and tissue fixation and staining were in accordance with procedures cited above.

The effect of fowl pox vaccination (stress) on the course of infectious hepatitis also was studied. In two separate and parallel groups of 7-week-old White Leghorn chickens, each bird, properly identified, was inoculated intramuscularly or orally with 0.1 ml of IHA inoculum. At the same time, the

Fig. 8. Photomicrograph of dilated tubules containing heterophilic casts in the kidney of a naturally infected mature chicken. A portion of a focus of heterophils is located in the upper right-hand corner (204×).

birds in one of the groups also were given fowl pox vaccine via the wing web. Uninoculated controls were kept in each trial. The chickens were necropsied two weeks later and were examined comparatively for lesions.

RESULTS

Signs of morbidity in chicks infected with the IHA (1297) usually were not pronounced, even in birds with severe lesions. There was some decrease in feed consumption. Mortality, which occurred early in the disease, did not exceed 5 percent. Gross tissue changes were seen in the spleens, livers or hearts or all three organs of young chickens as early as 48 hours post inoculation. The spleens were enlarged and pale during the early phase of the disease. The livers were enlarged up to twice normal size and had small grayish-white or yellowish-white areas of focal degeneration (figures 3, 9). Small areas of hemorrhage were seen through the liver capsule. Sometimes blood collected under the capsule to form red "bubble-like" distensions (figure 10). When distentions were excessive, the capsule burst spilling blood into the body cavity. The heart often was enlarged, pale and flaccid. Straw-colored fluid filled and sometimes distended the pericardial sac. Ascites was present in individuals with more pronounced lesions. The liver lesions were most prominent between 5 and 12 days post inoculation, whereas, the heart lesions persisted up to 6 weeks post inoculation. The kidneys sometimes appeared swollen and pale. The blood disturbances in chicks were most pronounced between the 2nd and 21st day post inoculation. The heterophil, thrombocyte, and total leukocyte counts increased. The hemoglobin content, hematocrit, erythrocyte and lymphocyte counts decreased.

In older birds experimentally inoculated, there was some unthriftiness but no mortality during the periods which they were held. Gross tissue changes were not seen until 5 to 15 days post inoculation at which time livers showed grayish-white pin-head size areas of degeneration. Heart lesions were less frequent and less pronounced than in chicks. Liver lesions persisted for at least 9 weeks post inoculation when the agent

Fig. 9. (top) Photograph of a liver of an experimentally infected chick with focal degeneration 11 days post inoculation.

Fig. 10. (bottom) Experimentally infected chick 7 days post inoculation. Photograph of liver with a subcapsular hematocyst.

Fig. 11. Experimentally infected chick 7 days post inoculation. Photomicrograph of cardiac muscle infiltrated by mononuclear cells (240×).

was still recoverable. Hens with more severe lesions were out of production.

With few exceptions, the microscopic appearance of these experimentally produced lesions were similar to the respective lesions in naturally infected young or mature birds. Inflammatory changes in the hearts of young birds were more severe than in older ones (figure 11). Inflammatory reactions and degenerations of the liver of experimental birds (figure 12) were similar to field cases except that granulomatous lesions were not seen in experimentally infected birds; neither were they seen in the spleens. Inflammatory reactions in the kidneys of experimental birds were less frequent and pronounced than in birds from field cases.

Fowl pox vaccination (stress) did not alter significantly the course of experimental infections with infectious hepa-

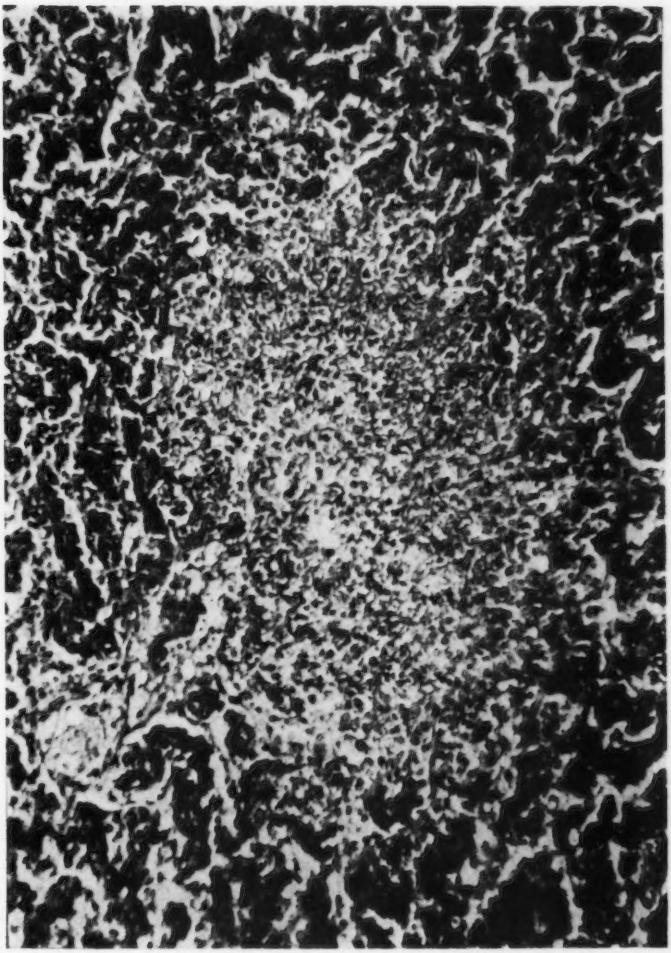


Fig. 12. Experimentally infected chick 7 days post inoculation. Photomicrograph of a liver section with focal degeneration (240×).

titis. The degree of severity and percentages of infection of groups orally or intramuscularly inoculated with IHA were comparable to similarly inoculated groups also given fowl

pox vaccine. A satisfactory "take" of fowl pox vaccination was evidenced by the presence of scab lesions at the administered site 10 days post inoculation.

DISCUSSION

A disease entity involving particularly the livers, hearts and spleens of chickens has been observed during the past several years in the Poultry Disease Diagnostic Laboratories in Massachusetts and elsewhere. The predominant lesions were inflammation, hemorrhage and degeneration in the liver, edema and mononuclear infiltration of the heart, and lymphoid cell proliferation followed by degeneration in the spleen. This disease entity does not appear to be the same as that described by Lukas[5] as a nonsuppurative hepatitis without hemorrhage or necrosis. On the other hand, the disease appears to be similar if not the same as the ones described by Delaplane *et al.*[2] and Tudor.[6]

Isolation attempts of the IHA using the techniques described above frequently were unsuccessful from field cases submitted to the laboratories where such cases were identified as infectious hepatitis from gross and histological lesions. When a group of chickens was submitted from a given flock suspected of being infected, all birds within the group were cultured routinely. From infected groups, isolations sometimes were successful from chickens having no gross lesions. It would appear that isolation attempts may be more successful when supernatant fluids from minced hearts, livers, or both, are used to inoculate 6-day-old embryonated chicken eggs than when suspended stab cultures are used for similar inoculations. The use of certain antibiotics[4] was helpful in minimizing contamination, particularly when tisssues from dead birds were cultured. Furthermore, their use may be helpful in differentiating the IHA from other infectious agents which are sensitive to these antibiotics.

The gross appearance of avian infectious hepatitis often resembled that of lymphomatosis, blackhead. blue comb, pullorum disease, fowl typhoid, or fowl cholera. Histopathological examinations, isolations of the casual agent with or

without the use of certain antibiotics, and reproduction of the disease in chickens have proved helpful to the workers in our laboratories in differentiating infectious hepatitis from other disease entities.

ACKNOWLEDGMENTS

Appreciation is expressed to Drs. G. P. Faddoul and G. H. Snoeyenbos for directing to the authors field material for investigation.

Costs of reproduction of colored prints were paid for, in part, by Eaton Laboratories, Norwich, N. Y.

SUMMARY

Clinical and pathological manifestations of a disease recognized most frequently as a primary hepatitis in chickens have been described. Numerous isolations from five different flocks have been made in Massachusetts.

The signs of the disease in chickens under field conditions usually were not pronounced and had an isidious onset. Egg production lagged where laying pullets were involved or decreased as much as 35 percent during variable periods of time in older flocks. Usually a small percentage of the birds in an affected flock appeared morbid at any one time, but the mortality in severely involved flocks ranged up to 15 percent during the course of several weeks.

On necropsy the most striking features of the natural disease in mature or nearly mature chickens were degenerative and hemorrhagic lesions in the liver. In younger chickens, the hearts were more severely and consistently involved. A straw-colored gelatinous exudate clung to the epicardium whereas a more watery material filled and sometimes distended the pericardial sac.

In mild or early infections, lymphocytic foci sometimes containing a few eosinophilic granulocytes were seen microscopically most often around the portal areas of the liver. In more severe cases, lymphocytic and granulocytic foci and focal degeneration were the predominant changes. There was a decrease in the lymphopoietic activity in the spleen during the advanced stages of the disease. A predominantly hetero-

philic inflammation was present in the kidneys. The bone marrow of affected birds had increased numbers of myelocytes. In young chickens, mononuclear cell infiltration of the epicardium and myocardium was more common and pronounced than in mature birds.

Experimentally, chicks appeared to be more susceptible to the IHA than older birds. In infected birds under controlled conditions, the heterophil, thrombocyte and total leukocyte counts increased; the hemoglobin content, hematocrit, erythrocyte and lymphocyte counts decreased.

Gross tissue changes were seen as early as 48 hours post inoculation in chicks, but not until 5 to 15 days post inoculation in older birds. With few exceptions, the lesions seen in experimental infections were similar to those of field infections.

In one trial, fowl pox vaccination (stress) did not alter significantly the course of experimental infectious hepatitis in chickens.

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AVIAN INFECTIOUS HEPATITIS. II. SOME CHARACTERISTICS OF THE ETIOLOGIC AGENT. EFFECT OF VARIOUS DRUGS ON THE COURSE OF THE DISEASE.*

By R. W. WINTERFIELD, M. SEVOIAN and C. L. GOLDMAN

Department of Veterinary Science
University of Massachusetts
Amherst, Massachusetts

Received 9 July 1957

INFECTIOUS hepatitis (IH) of chickens as a clinicopathologic entity has been described and the literature reviewed.[4] It was the purpose of this investigation to determine some of the properties of the causal agent and the effect of various antibiotic and chemotherapeutic drugs on the course of the disease.

MATERIALS AND METHODS

Four strains of the infectious hepatitis agent (IHA) were employed and identified as 9539, 1297, 9683, and 9805. Each was isolated from affected chickens in widely separated flocks (over 50 miles apart). The isolation of IHA 9539 and the technique used have previously been described.[5] Strains 1297 and 9683 were isolated in a similar manner from the livers of affected birds. However, 9805 was recovered from the pericardium and myocardium of 10-week-old pullets. The disease syndrome as it appears in the field has been discussed by the authors.[4,5]

Fertile hens' eggs and Rhode Island Red chickens for experimental inoculation were procured from a closed flock maintained at the University poultry farm. Initial isolations,

* Contribution 1110 of the Massachusetts Agricultural Experiment Station.

subsequent titrations, and serial passages (table I) were via yolk sac inoculations of 6-day-old embryos. On routine yolk sac passage, infective yolk was diluted 10^{-1} with sterile physiological saline prior to injection. When the agent was passed in the amniollantoic cavity of 10-day-old embryos (table I), however, undiluted yolk and allantoic fluid (approximately 1:1) was used. Regardless of the route, method of inoculation, or dilution factor, 0.1 ml of inoculum was delivered per egg. All inoculated embryos were candled daily and incubated until 20 or 21 days of age unless death occurred prior to this time. Deaths within the first 48 hours after inoculation were regarded as nonspecific. End points from titration of the agent were caculated by the method of Reed and Muench.[3] Five embryos per dilution were always used in the titrations.

Antiobiotic and chemotherapeutic drugs were employed in sensitivity trials (table II). The following were tested in various amounts against 10^4 embryo lethal doses (eld) per ml of each strain: chlortetracycline, oxytetracycline, magnamycin, furazolidone, dihydrostreptomycin sulfate, penicillin G potassium, chloramphenical, polymyxin B sulfate and bacitracin. Drug-IHA mixtures were incubated for 30 minutes at room temperature (25 C) prior to inoculation into the yolk sac of 6-day-old embryos.

The prophylactic effect of the following drugs was assessed in 2-day-old chickens (table III): chlortetracycline, oxytetracycline, furazolidone, dihydrostreptomycin sulfate, sulfaquinoxaline sodium and sulfamethazine sodium. Chickens were highly susceptible at this early age when inoculated via oral, subcutaneous, intramuscular or intraperitoneal routes with the IHA.[4,5] A satisfactory "take" was obtained when an inoculum passed a small number of times in eggs was given by the intraperitoneal route in a 0.1 ml amount per bird. Thus, strain 1297 in the second embryo passage was used. The experimental lots, with the exception of the controls, were placed on medicated feed or water at one day of age, inoculated with the IHA at 2 days and then necropsied 4 days later. When dihydrostreptomycin sulfate was given, it was administered intramuscularly to each bird at the time of IHA in-

jection. Previous studies[4] had indicated a short incubation period and rapid development of pathologic signs at this age. Therefore, the foregoing schedule was carried out. In trial 1 (table III), recovery of the IHA was attempted from individual birds of each lot at the time of necropsy. In trial 4 (table III), all lots of medicated feed had the drug withdrawn at 6 days of age. Approximately two-thirds of each lot were necropsied at 8 days of age and the remainder at 12 days, at which time IHA isolations from each chicken were attempted in an effort to determine the effect of drug withdrawal. When necropsied, all birds were individually scored for the presence, or absence, and severity of the gross, pathologic changes[4] characteristic of the disease: 0, no significant changes; 1, slight; 2, slight to moderate; 3, moderate to severe; and 4, severe. Individuals that died during the trial were given a score of 4.

The following drugs were given on a therapeutic basis (table IV): chlortetracycline, oxytetracycline, furazolidone, dihydrostreptomycin sulfate and nitrofurazone. The experimental design was similar to the prophylaxis trials with these exceptions: medication in the feed or water, where used, was commenced 24 hours after inoculation with the IHA and then withdrawn at the end of a 6 day period; where dihydrostreptomycin sulfate was given, it was administered intramuscularly 24 hours after IHA inoculation. After the medicated feed or water had been withdrawn for 4 days, all birds were necropsied and scored.

In filtration experiments, infective yolk of strain 1297 was diluted 1:10 with 0.85 percent saline with bacitracin and polymyxin added to give a final concentration of 120 and 1200 units respectively per ml. Hearts and livers from 11 and 18-day-old chickens that had been inoculated with the IHA at 2 days of age were finely chopped with scissors in the saline-antibiotic combination (approximately 1 part tissue to 10 parts saline). This suspension was centrifuged at 2000 revolutions per minute for 5 minutes, and the supernatant fluid was then used. These preparations were then filtered through Millipore membranes of different pore diameters.

Seitz pads and filters were also used after the yolk-saline suspension was passed through Whatman 3 filter paper. Filtrates and aliquots of the original material were inoculated into the yolk sac of 6-day-old embryos and cultured in aerobic and anaecrobic bacteriologic media. Subpassages were made with filtrates when necessary.

Yolk material was prepared in the same manner as for filtration and subjected to centrifugation at 27,500 times gravity for various intervals of time. The presence or absence of the IHA in the supernatant and sediment was then assessed by embryo inoculation.

Neutralization tests were performed on serums of 10½-week-old chickens which had been given IHA strain 9539. Two of the chickens were given infective yolk intravenously, and two through the intraperitoneal route at 6½ weeks of age and then injected again a week later. The same number of birds were given noninfective yolk in a similar manner and at the same time. Two chickens were retained as uninoculated controls. All serums were inactivated at 56 C for 30 minutes and used undiluted against serial, 10-fold dilutions of IHA strain 9539. The presence or absence of IH neutralizing antibodies was then determined in 6-day-old chicken embryos.

Host susceptibility tests were conducted with day-old Broad Breasted Bronze turkey poults, New Zealand white rabbits (4 to 5 weeks of age), Swiss albino mice (10 to 12 days of age), and adult hamsters. Inoculations were by intraperitoneal, intramuscular, or oral routes using IHA strains 9539 and 1297 in second embryo passages. If death did not intervene, necropsies and IHA recovery attempts were made 7 to 11 days after inoculation.

The distribution of IHA in the blood and various tissues of infected chickens was determined by inoculating day-old chickens (intraperitoneal route) with strain 1297 and then titrating pooled blood and tissues from four to eight birds at 5 and 12 days post inoculation.

RESULTS

Propagation of the agent in chicken embryos. Table 1 indicates the death pattern from representative passages of

TABLE I

Death pattern of chicken embryos resulting from the propagation of the infectious hepatitis agent in the yolk sac (ys) and amnioallantoic cavity (aac)

Strain of agent	Passage no.	Route of inoculation	Embryo age (days)	No. of embryos	Days post inoculation and embryo deaths														Log eld_{50}/ml of passage pool
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
9539	1	ys	6	10				8	2											4.6
	2	ys	6	10			9	1												5.3
	3	ys	6	10	1		9													5.6
	8	ys	6	10	1		9													6.5
	20	ys	6	5		1	4													6.5
9539	2	aac	10	10					3	5	2									—
	5	aac	10	10						1		3	2	1	3					—
	13	aac	10	10	1		1	1	3	2	2									5.2
	20	aac	10	8	1			3	3	1										—
1297	1	ys	6	6	1				4	1										—
	2	ys	6	8			5	3												—
	7	ys	6	5	1		3										1			5.3
	10	ys	6	5	1		4													—
1297	3	aac	10	10	1					1		2	3	3						—
	5	aac	10	10						1	2	2	3	1	1					3.8
	10	aac	10	8	1				2	3	2									—
9683	1	ys	6	8				6	2											—
	5	ys	6	5	1		4													4.5
	10	ys	6	5			5													6.5
9683	2	aac	10	10	1	2		2	2	1									2	—
	5	aac	10	8	1				2	3			2							—
	10	aac	10	8		1	1	1		5										—

three IHA strains in the yolk sac and amnioallantoic cavity. On initial isolation from livers or hearts, death of one or more embryos generally occurred from 4 to 6 days post inoculation (yolk sac), while on 2nd passage all died within 3 to 4 days. Subsequent passage of the agent invariably resulted in embryo death on the 3rd day. When the agent was propagated in 10-day-old embryos (amnioallantoic cavity), deaths were frequently delayed and occurred from 3 to 11 days after inoculation. The agent was propagated serially by both routes without difficulty.

Titrations of harvested yolk material pools from dead embryos that had been inoculated via the yolk sac showed yields of the agent varying between $10^{3.8}$ and $10^{6.5}$ eld_{50} per ml. After the 2nd and subsequent yolk sac passages of each strain, titers of $10^{4.5}$ to $10^{6.5}$ were routinely recorded. Separate harvests and titrations of yolk and allantoic fluid from the same infected embryo revealed none of the IHA in the allantoic fluid in the majority of trials. In an exceptional case, IHA was detected in the allantoic fluid at very low titers (10^{1} to 10^{2}). The high concentration of the IHA in the yolk versus little or none in allantoic fluid was observed regardless of whether the embryos had been inoculated via the yolk sac or amnioallantoic cavity.

Attempts at isolating the agent on artificial media from infective yolk of various passages and affected livers met with negative results when the following were used: thioglycollate broth, thioglycollate broth + 5 to 40 percent horse serum, thioglycollate broth + 5 to 40 percent yolk, CPLM and trypticase with 10 percent horse serum (for protozoa), beef extract broth, PPLO broth and agar (Difco), and blood and chicken infusion agars.

On serial yolk sac passage of the IHA, virulence for day-old chicks progressively decreased. Whereas strain 9539 was highly pathogenic after the 1st and 2nd passage, it was only slightly virulent at the 8th and had become completely apathogenic at the 20th passage level. Strain 1297 likewise followed a similar pattern of decreased virulence after repeated egg passage. Strains 9683 and 9805 were pathogenic after the first and second embryo passages, but subsequent generations were not tested.

Sensitivity of the IHA to various drugs. Table II summarizes the results obtained with strain 9539. Chlortetracycline, oxytetracycline, magnamycin and furazolidone gave complete protection of the embryos from death. Good protection was also afforded by dihydrostreptomycin sulfate. Penicillin G potassium had a slight inhibitory action on the IHA as evidenced by survival of one third to two thirds of the embryos, depending on the amount of antibiotic used, while all the infected controls died. Chloramphenicol delayed embryo death, but all eventually died. Polymyxin B sulfate and bacitracin at the concentrations employed failed to demonstrate inhibitory action. Parallel inoculations did not result in a toxic effect on the embryos with the drugs and quantities used in these trials, and in the absence of the IHA.

The other three strains employed in sensitivity trials gave results similar to the foregoing with the exception of 9805. When the latter strain was used in the presence of chlortetracycline, the number of embryo deaths exceeded 50 percent thereby suggesting a possible strain resistance to this antibiotic. However, all were sensitive to oxytetracycline, magnamycin, furazolidone and dihydrostreptomycin sulfate. Again penicillin G potassium delayed embryo death in each instance, and chloramphenicol completely failed to protect. Inasmuch as polymyxin or bactracin had no apparent effect on the agent, a combination of the two antibiotics was used when isolations were attempted from partially decomposed dead birds and in situations where contamination of IHA cultures was likely during the necropsy and culturing of large numbers of chickens in prophylaxis and therapy trials. This combination appeared satisfactory in inhibiting many Gram-negative and Gram-positive contaminants occasionally encountered in routine diagnostic work.

Prophylactic effect of various drugs on the disease. Table III summarizes the data from five trials. Chlortetracycline, oxytetracycline and furazolidone significantly suppressed development of the gross pathologic lesions associated with IH when each was administered at levels of 0.022, 0.033 and 0.044 percent in the feed (200, 300 and 400 gm per ton respectively) in trials 1, 2 and 4. However, at 0.0055 and 0.011

TABLE II

Drug sensitivity of the infectious hepatitis agent when assayed in 6-day-old chicken embryos

Drug assayed	Amt.* of drug/ml of inoculum†	Days post inoculation and embryo deaths															No. deaths/No. inoculated
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Chlortetracycline	5 milligrams																0/6
Chlortetracycline	2.5 milligrams																0/6
Oxytetracycline	5 milligrams																0/6
Oxytetracycline	2.5 milligrams																0/6
Magnamycin	5 milligrams																0/6
Furazolidone	1 milligram																0/6
Dihydrostreptomycin sulfate	5000 units										1						1/6
Dihydrostreptomycin sulfate	2500 units										1						1/6
Penicillin G potassium	5000 units				1	1	1			1							4/6
Penicillin G potassium	10,000 units					1		1									2/6
Chloramphenicol	5 milligrams				1	4		1									6/6
Chloramphenicol	10 milligrams	1					3	1	1								6/6
Polymyxin B sulfate	5000 units				3	2	1										6/6
Polymyxin B sulfate	10,000 units	1			1	4											6/6
Bacitracin	500 units				6												6/6
Bacitracin	1000 units	1		2	3												6/6
Infected-untreated controls				4	2												6/6
Uninfected-untreated controls																	0/6

* Amounts tested were nontoxic for embryos in parallel trials.

† 10,000 embryo letthal doses of IHA strain 9539 per ml.

percent levels (50 and 100 gm per ton respectively) in trial 3, there was apparently little or no inhibition. Dihydrostreptomycin sulfate given intramuscularly (5 mg per bird) also gave favorable suppressive action (trials 4 and 5). The IHA was not recovered on isolation attempts at necropsy from any of the birds on feed medication in trial 1 indicating an inhibition of the multiplication of the agent. On the other hand, recovery attempts were successful from 75 percent of the infected, unmedicated controls.

In trial 4, where the feed medication had been withdrawn at 6 days of age and two thirds of the chickens were necropsied 2 days later the severity and extent of gross pathologic lesions were substantially lessened in the medicated lots, as indicated in the table. When the remainder of the birds in this trial were necropsied at 12 days of age (6 days after medicated feed withdrawal and 10 days after injection with dihydrostreptomycin sulfate), the percent of each lot showing pathologic signs of the disease was as follows: chlortetracycline, 30.8 percent; oxytetracycline, 83.3 perecent; furazolidone, 0.0 percent; dihydrostreptomycin sulfate, 0.0 percent; infected controls, 100 percent; and uninfected controls, 0.0 percent. IHA recoveries from each lot at this time were as folows: chlortetracycline, 38.4 percent; oxytetracycline, 83.3 percent; furazolidone, 0.0 percent; dihydrostreptomycin sulfate, 0.0 percent; infected controls, 50 percent; and uninfected controls, 0.0 percent.

In the majority of trials with levels of 0.022, 0.033, and 0.044 percent of the tetracyclines and furazolidone in the feed, furazolidone showed the greatest suppressive action on the course of the disease. Apparently there was no tendency towards a relapse (trial 4) after furazolidone had been withdrawn for 6 days, and IHA was not isolated from any of the chickens. Likewise, dihydrostreptomycin sulfate showed good prophlactic action. Evidence of a relapse was lacking, and the IHA was not recovered. In contrast, those lots on the tetracycline antibiotics showed a greater proportion of the birds with gross IH lesions after the extended drug withdrawal period. Furthermore, a high isolation rate of the IHA

TABLE III

Prophylactic effect of various drugs on infectious hepatitis of chickens

Trial no.	Drug	Amt. of drug and method administered	No. of chickens per lot	% showing gross lesions at necropsy or death	Severity of lesions at necropsy or death with score of 0 to 4* no. of birds/score
1	Chlortetracycline	0.044% in feed	26	7.8	24/0 and 2/3
	Chlortetracycline	0.022% in feed	26	15.4	22/0 and 4/1
	Oxytetracycline	0.044% in feed	30	0.0	30/0
	Oxytetracycline	0.022% in feed	24	4.2	23/0 and 1/1
	Furazolidone	0.044% in feed	33	0.0	33/0
	Furazolidone	0.022% in feed	28	17.8	23/0, 4/1 and 1/3
	Infected controls	———	34	73.5	9/0, 13/1, 5/2, 5/3 and 2/4
	Uninfected controls	———	24	0.0	24/0
2	Chlortetracycline	0.033% in feed	26	26.9	19/0 and 7/1
	Chlortetracycline	0.022% in feed	25	24.0	19/0 and 6/1
	Oxytetracycline	0.033% in feed	25	28.0	18/0, 6/1 and 1/2
	Oxytetracycline	0.022% in feed	26	30.8	18/0, 7/1 and 1/2
	Furazolidone	0.033% in feed	25	12.0	22/0 and 3/1
	Furazolidone	0.022% in feed	26	19.2	21/0 and 5/1
	Infected controls	———	26	80.0	5/0, 14/1, 4/2 and 2/3
	Uninfected controls	———	23	0.0	23/0
3	Chlortetracycline	0.011% in feed	24	75.0	6/0, 16/1, 1/2 and 1/3
	Chlortetracycline	0.0055% in feed	24	87.5	3/0, 14/1, 5/2 and 2/3
	Oxytetracycline	0.011% in feed	23	52.2	11/0, 10/1 and 2/3
	Oxytetracycline	0.0055% in feed	25	91.7	2/0, 15/1, 4/2, and 3/3
	Furazolidone	0.011% in feed	22	59.1	9/0, 8/1, 2/2 and 3/3
	Furazolidone	0.0055% in feed	23	78.7	5/0, 13/1, 3/2, 1/3 and 1/4
	Infected controls	———	26	88.5	3/0, 10/1, 4/2, 6/3 and 3/4
	Uninfected controls	———	24	0.0	24/0

* 0, no significant changes; 1, slight; 2, slight to moderate; 3, moderate to severe; and 4, severe. Chickens that died during the trial were given a score of 4.

TABLE III (Continued)

Prophylactic effect of various drugs on infectious hepatitis of chickens.

Trial no.	Drug	Amt. of drug and method administered	No. of chickens per lot	% showing gross lesions at necropsy or death	Severity of lesions at necropsy or death with score of 0 to 4* no. of birds/score
4	Chlortetracycline	0.044% in feed	21	23.8	16/0 and 5/1
	Oxytetracycline	0.044% in feed	21	47.7	11/0, 9/1 and 1/2
	Furazolidone	0.044% in feed	20	10.0	18/0 and 2/1
	Streptomycin	5 mg. i.m.	20	5.0	19/0 and 1/1
	Infected controls	———	21	100.0	10/1, 5/2, 1/3 and 5/4
	Uninfected controls	———	22	0.0	22/0
5	Sulfamethazine	0.10% in water	25	25.0	20/0, 4/1 and 1/2
	Sulmaquinoxaline	0.04% in water	23	30.4	16/0, 6/1 and 1/3
	Streptomycin	5 mg. i.m.	25	4.0	24/0 and 1/1
	Infected controls	———	22	95.5	1/0, 5/1, 5/2 and 11/4
	Uninfected controls	———	15	0.0	15/0

* 0, no significant changes; 1, slight; 2, slight to moderate; 3, moderate to severe; and 4, severe. Chickens that died during the trial were given a score of 4.

was obtained. It was thus suggested that the pathogenesis of the disease was inhibited during chlortetracycline and oxytetracycline administration and that a possible relapse had ocurred after antibiotic withdrawal.

Although sulfamethazine and sulfaquinoxaline showed inhibitory action on the course of the disease (trial 5), the results were not so satisfactory as with dihydrostreptomycin sulfate. The majority of the birds appeared debilitated after the period of sulfonamide medication and did not possess the fleshing and vigor of the antibiotic-treated chickens. It is of interest that IHA strain 9805 was isolated from 10-week-old pullets that were being administered sulfaquinoxaline as a coccidiostat on a prophylactic basis (0.0175 percent level) in the feed.

Therapeutic effect of various drugs on the disease. Table IV indicates the results from 4 trials. It is evident that furazolidone was considerably more effective than chlortetracycline and oxytetracycline at the 0.044 percent level (400 gm per ton) in the feed (trial 1). Similar results were also obtained when furazolidone in the feed was compared with the soluble tetracycline antibiotics administered in the drinking water (trail 2). The data indicate that chlortetracycline and oxytetracycline had little or no therapeutic value.

While dihydrostretptomycin sulfate proved superior to chlortetracycline and oxytetracycline (trials 1 and 2), it was consistently inferior to furazolidone administration (trials 1, 2, 3 and 4) in altering the course of the disease. Moreover, furazodilone was highly effective at three different levels (trials 3 and 4) : 0.022 percent (200 gm per ton), 0.033 percent (300 gm per ton) and 0.044 percent (400 gm per ton). Soluble nitrofurazone, while having some effect on the disease (trials 3 and 4), proved unsatisfactory as compared to furazolidone.

In an effort to determine if a relapse would occur after furazolidone withdrawal, chickens were isolated with the IHA maintained as in trials 1, 2, 3 and 4. Infected birds (approximately 50 per lot) were treated with 0.022, 0.033 and 0.044 percent levels of the drug in the feed for 6 days; 25

TABLE IV

Therapeutic effect of various drugs on infectious hepatitis of chickens

Trial no.	Drug	Amt. of drug and method administered	No. of chickens per lot	% showing gross lesions at necropsy or death	Severity of lesions at necropsy or death with score of 0 to 4 * no. of birds/score
1	Chlortetracycline	0.044% in feed	34	91.2	3/0, 9/1, 12/2, 7/3 and 3/4
	Oxytetracycline	0.044% in feed	32	100.0	22/1, 3/2, 4/3 and 3/4
	Furazolidone	0.044% in feed	33	6.0	31/0 and 2/1
	Streptomycin	5 mg. i.m.	33	66.6	11/0, 13/1, 5/2 and 4/3
	Infected controls	———	33	100.0	22/1, 9/2 and 2/3
	Uninfected controls	———	32	0.0	32/0
2	Chlortetracycline	0.022% in water	29	79.3	6/0, 10/1, 8/2, 3/3 and 2/4
	Oxytetracycline	0.022% in water	29	93.1	1/0, 10/1, 12/2 and 6/3
	Furazolidone	0.044% in feed	25	8.0	23/0 and 2/1
	Streptomycin	5 mg. i.m.	28	25.0	21/0, 6/1 and 1/2
	Infected controls	———	29	100.0	5/1, 17/2, 6/3 and 1/4
	Uninfected controls	———	27	0.0	27/0
3	Furazolidone	0.044% in feed	28	3.6	27/0 and 1/1
	Furazolidone	0.033% in feed	27	7.4	25/0 and 2/1
	Furazolidone	0.022% in feed	28	10.7	25/0 and 3/1
	Nitrofurazone	0.022% in water	25	84.0	4/0, 6/1, 4/2, 10/3 and 1/4
	Nitrofurazone	0.011% in water	25	84.0	4/0, 5/1, 9/2, 6/3 and 1/4
	Streptomycin	5 mg. i.m.	28	35.7	18/0, 7/1, 1/2 and 2/3
	Infected controls	———	20	95.0	1/0, 9/1, 6/2 and 4/4
	Uninfected controls	———	28	0.0	28/0
4	Furazolidone	0.044% in feed	34	11.7	30/0 and 4/1
	Furazolidone	0.033% in feed	27	7.4	25/0 and 2/1
	Furazolidone	0.022% in feed	27	11.1	24/0 and 3/1
	Nitrofurazone	0.022% in water	26	23.1	20/0, 5/1 and 1/3
	Nitrofurazone	0.011% in water	27	100.0	6/1, 8/2, 9/3 and 4/4
	Streptomycin	5 mg. i.m.	31	35.5	20/0 and 11/1
	Infected controls	———	22	95.4	1/0, 9/2, 8/3, and 4/4
	Uninfected controls	———	19	0.0	19/0

* 0, no significant changes; 1, slight; 2, slight to moderate; 3, moderate to severe; and 4, severe. Chickens that died during the trial were given a score of 4.

were left as infected, untreated controls and 25 as uninfected controls. Necropsies were made of randomly selected birds over a 3-week period after discontinuing treatment. The results again demonstrated the favorable action of furazolidone and there was no evidence of a relapse. Whereas some of the infected, untreated controls made an apparent recovery, others still showed signs of the disease at the end of the observation period. None of the furazolidone-medicated birds showed gross pathology suggestive of IH.

In an extension of trial 5 and at the end of the 3-week observation period, an attempt was made to reinfect chickens remaining in the lot which had been medicated at the 0.022 percent level of furazolidone. At this time, 19 birds from this lot were necropsied and none showed gross pathologic lesions suggestive of IH. Ten chickens from the group were inoculated by the intraperitoneal route with the same strain (1297) of IHA that had originally been given. When necropsied 11 days later, five out of 10 showed extensive pathologic lesions of IH whereas five uninoculated controls from the same lot showed no signs of hepatitis. Of five birds having no known contact with IHA, all showed signs of infection after inoculation. Again five uninoculated controls remained negative. The presence or absence of infection in the various lots was confirmed by individual IHA isolation attempts in 6-day-old chicken embryos. Thus it appeared that little or no resistance to infection by the agent had developed.

Filtration. When fluids from macerated, infected chicken heart-liver suspensions containing high concentrations (10^6/ml) of the agent were employed, the IHA readily passed 1.2, 0.8 and 0.45 micron membrane Millipore filters, but not 0.30. Filtration attempts with infective yolk, however, were generally unsatisfactory because of the high lipid and protein levels present. While the agent passed 5.0 and 2.0 micron Seitz pads and filters, it did not pass the EK. Observations were inconclusive on Macchiavello, Giemsa and Gram-stained infectious material when compared to stained, uninfected control preparations. Aerobic and anaerobic cultures of filtrates for known bacterial agents were negative.

Centrifugation. When 10 percent yolk suspensions of the IHA were centrifuged at 27,500 times gravity for 20 or more minutes, the agent was completely sedimented. However, at 15 minutes and less the supernatant material remained infective.

Serum neutralization tests. Neutralizing antibodies could not be demonstrated in the serums of experimentally infected chickens. Neutralization indices were all < 1.0 in birds inoculated twice with IHA strain 9539. The presence of active infection was shown by pathologic evidence of the disease in the birds 4 weeks post inoculation and recovery of the IHA. Uninfected controls and controls inoculated with normal yolk were negative for neutralizing antibodies, did not show evidence of the disease, and failed to yield the IHA agent.

Host susceptibility. Day-old turkey poults were infected with IHA in each of two trials when the inoculum was administered by the intramuscular or intraperitoneal route. The agent was recovered from affected livers. Negative results were obtained, however, when the agent was given orally in one of the trials. Although turkeys proved susceptible to infection, the pathologic changes were never so severe or extensive as those observed in chickens after inoculation with material from the same infective yolk pool.

In one of two trails, 4-week-old rabbits were infected with strain 1297 administered by the intraperitoneal route. One rabbit died of acute illness 5 days post inoculation, and the agent was recovered from the liver. The use of two different pools of the IHA in these trials may account for the negative results from the one experiment.

Negative results were obtained from intraperitoneal mouse inoculations from four trials with early egg-passaged material of strains 9539 and 1297. No clinical evidence of illness was seen, and no IHA isolations were made on necropsy 11 days after inoculation. Infection of adult hamsters could not be demonstrated under similar circumstances.

Distribution of the agent in the chicken. Five days post inoculation IHA isolation attempts were made, and the follow-

ing yields were obtained (log e.l.d.$_{50}$ per gm of tissue or ml of blood): heart, 6.5; liver, 5.7; blood, 4.4; kidney, 3.8; and spleen, 3.5. Concentrations of the agent from birds necropsied at 12 days were as follows: heart, 5.8; liver, 5.4; blood, 2.6; kidney, 2.2; and spleen, 1.8.

Viability of the agent. When livers of affected birds obtained from field outbreaks were held in a mechanical freezer at —20 C, the agent (9539) was recoverable in embryos until the 13th day of storage but not on subsequent isolation attempts. On the other hand, livers and hearts stored at — 56 C yielded the agent after 28 days (longest period tested at this temperature). Livers kept in an unfrozen state at 4 C yielded the agent (9683) until the 7th day of storage but not on the 13th.

Yolk material of strain 9539, 1st embryo passage, was still infective for embryos after 5 months storage at — 20 C and — 56 C. Yolk material harvested from dead embryos on the 2nd passage was infective and pathogenic for 2-day-old chicks. IHA strain 1297 was still viable after 4 months storage at these temperatures. When infective yolk (9539) was held at 37.5 C, it was viable at the 14th day but not at the 21st. At room temperature (25 C), strain 9683 retained its infectivity for embryos for 12 days but no longer. The higher the titer per ml of yolk upon entering storage, the greater were the chances of recovering the agent at a subsequent date. A similar observation was made with infective liver material of field origin.

DISCUSSION

The agent causing a primary hepatitis of chickens in this study does not appear to be the same as that described by Lukas.[2] On the other hand, it has many similarities to the agent described by Delaplane *et al.*[1] Some differences exist, however, which may be due to the technique. Also, differences in strains, or the agents themselves, must be considered. Although Delaplane *et al.*[1] were unable to propagate the agent serially in 10 and 11-day-old chicken embryos, three strains were passaged serially for as many as 20 generations

in this investigation. In our studies, combined yolk-allantoic fluid (about 1:1) was harvested from dead embryos and used undiluted for each successive passage into the amnio-allantoic cavity of 10-day-old embryos. Yolk material, containing the agent, was difficult to harvest from older embryos; therefore, it was released into the allantoic fluid, and this mixture was then drawn off with a syringe and needle. While this method proved successful, passage of allantoic fluid alone resulted in loss of the agent in one or two passages.

After initial isolation of the IHA from livers or hearts, the agent was readily adapted to propagation in the yolk sac of 6-day-old embryos on subsequent passage and then invariably had a lethal effect in dilutions greater than 10^{-3}. The agent of Delaplane *et al.*[1] failed to show 50 percent lethality in dilutions greater than 10^{-3}. Seven-day-old embryos were used for the most part in their studies.

Under the conditions of this study, the four strains of IHA tested were sensitive to oxytetracycline, magnamycin, furazolidone and dihydrostreptomycin sulfate. Three of the four strains were sensitive to chlortetracycline. All were slightly sensitive to penicillin G potassium in the concentrations used but relatively insensitive to chloramphenicol, polymyxin B sulfate and bacitracin. Delaplane *et al.*[1] reported that their agent was resistant to chlortetracycline, oxytetracycline and penicillin but was affected adversely by streptomycin in embryo assays. However, our studies suggest that there may be strain sensitivity differences, e.g., resistance of 9805 to chlortetracycline.

When chlortetracycline, oxytetracycline, furazolidone and dihydrostreptomycin sulfate were used prophylactically against the disease in chickens, the results were apparently in agreement with the embryo assays. Furazolidone and dihydrostetraptomycin sulfate gave superior results over the other drugs. Although the tetracyclines suppressed multiplication of the agent, it appears that a relapse may have occurred on withdrawal of these antibiotics. However, there was no evidence of a relapse where furazolidone or dihydrostretptomycin sulfate was administered.

When given on a therapeutic basis, furazolidone was con-

sistently better than any of the other drugs tested. Although dihydrostreptomycin sulfate was somewhat effective, the individual administration of the antibiotic is time-consuming and involves considerable labor expense where large, commerical laying flocks are involved. Moreover, the handling of birds in "lay" may have a deleterious effect on production. Limited field observations suggest that affected flocks respond well to furazolidone when given at 0.022 to 0.044 percent levels in the feed. However, in the absence of untreated controls, these observations must be cautiously interpreted. Further use and evaluation of furazolidone at different levels under field conditions would seem advisable in view of the encouraging results obtained in the laboratory screening experiments. The level of drug used and the period of medication may well be conditioned by the extent and severity of the disease in the individual flock. Possibly drug toxicity accompanying prolonged medication at the higher levels should be carefully evaluated.

To give a comparable dosage of dihydrostreptomycin sulfate used in the laboratory trials with chicks, 200 to 300 mg of the antibiotic should be administered intramuscularly per adult bird. In one field flock, a satisfactory response to streptomycin was observed, but the birds apparently suffered a relapse or re-infection during the year. Whether any degree of immunity or resistance to the disease results from active infection remains to be determined. It appears, however, that specific neutralizing antibodies were not formed following experimental infection. Furthermore, experimentally infected chickens that demonstrated apparent recovery after treatment with furazolidone were reinfected wtih the same IHA strain. Therefore, the possibility of a relpase or re-infection should be considered regardless of the therapeutic measures instituted or method of handling an infected flock.

The filtration studies suggest that the particle size of the IHA may be between 280 and 470 millimicrons. Although large viruses of the psittacosis-lymphogranuloma group fall within this range, inconclusive observations of Macchiavello and Giemsa-stained infectious material were made when com-

pared with noninfected control preparations.

In attempts at recovering the IHA from affected birds, the heart often yielded the agent in as high (if not higher) a concentration as did the liver. This was particularly true with chickens affected with the disease at a relatively early age. In the early stages of infection, the disease seemed to be of a systemic or septicemic nature. When infected livers or hearts were held at freezer temperatures of about — 20 C until such time as recoveries could be attempted, the chances of isolation of the agent were considerably lessened. On the other hand, when similar tissues were quickly frozen and held under dry ice storage, the frequency of isolation was enhanced.

ACKNOWLEDGMENT

The authors gratefully acknowledge the following firms for the drugs supplied in this investigation: Chas. Pfizer and Company, oxytetracycline, bacitracin and antibiotic diagnostic testing kits; American Cyanimid Company, chloretracycline; Burroughs Wellcome and Company, polymyxin B sulfate; Hess and Clark, furazolidone and nitrofurazone.

SUMMARY

Some characteristics of an infectious agent (four strains) causing a primary hepatitis in chickens have been described. The prophylactic and therapeutic effects of various drugs on the course of the experimentally induced disease have been determined.

1. The agent was readily propagated, and had a lethal effect, in 6-day- and 10-day-old chicken embryos inoculated via the yolk sac and amnioallantoic cavities, respectively. Although yolk from dead embryos gave high yields of the agent, the allantoic fluid showed little or none. Serial passage of the agent in embryos resulted in a progressive decrease in virulence of the agent for chickens. Routine aerobic and anaerobic cultures of infective yolk and affected livers were negative for known avian bacterial pathogens.

2. Under the conditions of this study when chicken embryos were used for assay, four strains of the agent were

sensitive to oxytetracycline, magnamycin, furazolidone and dihydrostreptomycin sulfate. Three of the four strains were sensitive to chlortetracycline. Penicillin G potassium had a slight inhibitory action, whereas polymyxin B sulfate, bacitracin and chloramphenicol gave no protection against embryo death with each of the strains employed.

3. A prophylactic effect on the course of the disease in chickens was demonstrated by the following drugs: chlortetracycline, oxytetracycline, furazolidone, dihydrostreptomycin sulfate, sulfamethazine, and sulfaquinoxaline. Furazolidone and dihydrostreptomycin sulfate proved most effective. A tendency towards a relapse was noted when the tetracycline antibiotics were withdrawn.

4. When adminstered therapeutically, furazolidone was highly effective in altering the course of the disease in chickens and was somewhat superior to dihydrostreptomycin sulfate. Chlortetracycline, oxytetracycline and soluble nitrofurazone were relatively ineffective.

5. The agent was readily filtered through 1.2, 0.8 and 0.45 micron membrane Millipore filters but did not pass the 0.30 membrane or the Seitz EK pad. In the form of a 10 percent yolk suspension, it was sedimented between 15 and 20 minutes after centrifuging at 27,500 times gravity.

6. Serum-neutralizing antibodies in chickens were not demonstrated following two injections of the agent. Infected chickens demonstrating apparent recovery after treatment were reinfected with the same strain of agent.

7. Infection of day-old turkey poults and 4 to 5-week-old rabbits with the agent was accomplished, whereas demonstration of infection in 10 to 12-day-old mice and adult hamsters failed.

8. In experimental infections of chickens, the agent was recovered from the heart, liver, blood, kidney and spleen.

9. Infective yolk yielded the agent after 5 months storage at — 20 C and — 56 C, 12 days at 25 C and 14 days at 37.5 C. Infected liver tissue when held at 4 C and — 20 C yielded the agent at the seventh and thirteenth day, respectively, of storage but not thereafter. The agent was isolated

from hearts and livers stored at — 56 C for 28 days (longest period tested at this temperature).

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STUDIES OF AN AGENT CAUSING HEPATITIS IN CHICKENS*

By RICHARD W. MOORE

Dekalb Agricultural Association Inc.
Dekalb, Illinois

Received 15 Aug 1957

A DISEASE characterized by hepatitis first attracted attention in 1953 when it was mistaken for fowl typhoid. After unsuccessful attempts to isolate *Salmonella gallinarum*, liver material was inoculated into chicken embryos and an agent was isolated[1]. Other workers[5,7,11] have reported similar syndromes. Some have isolated infectious agents, others could not.

* Part of this paper was submitted as a Master's thesis by the author in partial requirement for the Master of Science Degree in Veterinary Pathology at the Agricultural and Mechanical College of Texas.

The disease has occurred under field conditions only in a particular inbred line of chickens. The syndrome described by Tudor[11] may constitute an exception to this statement. The stress of egg production apparently contributes to the mortality, but the disease also occurs in males and non-producers to a limited extent. Genetic factors apparently influence the incidence of the infection because outcrosses from the inbred line remain free of the disease. Mortality averages about one percent every 10 days extending over a three to four month period in this inbred line.

The pathognomonic gross lesions occur in the liver. Ascites, pericarditis, and icterus are sometimes seen but are not constant lesions. Usually the birds are in a good state of nutrition at the time of death, but the birds that have ascites and icterus are those that are often in poor condition.

The first report of a comparable form of hepatitis in chickens was by Marthedal,[7] who described a syndrome presumably of non-contagious nature in chickens under three months of age. At necropsy the liver was enlarged and reddish-brown in color. Necrotic areas were scattered over the surface of the liver. The smallest were barely discernible and the largest were one to three mm in diameter. In some cases, the necrotic areas coalesced to a size of one to two cm in diameter. After limited virological and bacteriological studies, Marthedal attributed the condition to a nutritional deficiency but did not conclusively prove it.

Tudor[11]described a condition in which the livers were mottled and injected with color varying from a light yellowish-brown to a deep reddish-black. Tudor described the lesions as "stellate white flecks" diffusely scattered over the surface of some livers, whereas others had large "irregular hemorrhagic blotches". The liver tissue was usually very friable. No etiological agent was incriminated after various bacteriological and virological studies were undertaken. Studies of pictures of gross specimens and microscopic sections furnished by Tudor indicated similarity of this condition to that described by Delaplane, Smith, and Moore[1].

Lukas[5] described an infectious hepatitis caused by a fas-

tidious, pleomorphic, gram-negative organism coccoid or yeast-like in morphology. Injection of streptomycin was effective in controlling an outbreak. The agent described by Lukas grew in cell-free media, which is different from the growth of the agent isolated by Delaplane, *et al.*[1] This indicates there is probably no similarity between the two disease entities.

Delaplane, Smith, and Moore[1] described an agent that primarily caused a hepatitis in adult hens. This agent killed chicken embryos in four days on initial isolation and, as it became adapted to the chicken embryo, it killed in a somewhat shorter period. Day-old chicks from various sources when inoculated with this material had no noticeable symptoms, but when these chicks were killed at ten days of age, lesions were observed in the heart and liver. However, other chicks inoculated at one day of age had no gross lesions when necropsied three weeks later. This apparent recovery prompted the studies of immunity on large numbers of chicks and chickens to see if the incidence under field conditions could be reduced.

MATERIALS AND METHODS

Immunity studies. Seven-day-old chicken embryos were inoculated with 0.2 ml of the active agent. When these embryos died, the embryo, membranes, and the liquid portion of the egg were harvested. Each of the different components were pooled and ground in a Waring Blendor until a homogeneous mixture was obtained. The material was checked for sterility, bottled in sterile 500 ml serum bottles and stored at —20 C. Each lot was inoculated into ten one-day-old chickens to check for potency. This material was used as an inoculum on five separate lots of birds.

On March 21, 1954, 1000 one-day-old pullets (Group I inoculates) of the affected inbred line received subcutaneously 0.4 ml of the prepared inoculum. These birds were housed on farm A. One thousand pullets of the affected strain (Group I controls) were raised in separate pens as controls. On March 30, 1954, twenty chicks of the inoculated group were killed and examined for gross lesions. Weight records were maintained on representative groups of inoculated and control

birds for the first four weeks. All birds that died were preserved by freezing and when several birds had accumulated they were thawed and necropsies were performed. Records were kept of the date the bird died and the post mortem diagnosis on each lot until April 15, 1955.

On March 20, 1955, 1000 susceptible female chicks (Group II inoculates) and 100 susceptible male chicks at farm A were given 0.4 ml subcutaneously of the freshly prepared inoculum. One thousand susceptible female chicks (Group II controls) served as controls. On March 30, 1955, the 100 male chicks were killed and necropsies were performed to obtain an index of the infectivity of the inoculum. Beginning June 1, 1955, necropsies were performed upon all birds that died, and records were kept on each lot. On August 24, 1955, the pullets were given a second intraperitoneal inoculation using 0.5 ml of a freshly prepared inoculum. Data were collected through March 31, 1956, on this group of birds.

On March 5, 1955, three groups of susceptible birds, farm B, Group III—1000 two-week-old chickens, Group IV—500 five-week-old birds, Group V—500 eight-week-old birds, were injected subcutaneously with 0.4 ml of a freshly prepared inoculum. On August 30, 1955, these birds were again exposed to the agent in question by receiving 0.5 ml of egg material intraperitoneally. Necropsies were performed on all birds and records kept on the number of "hepatitis" cases as judged by gross lesions. Uninoculated controls were maintained for each group of the same breeding.

Studies of recovered birds. Since there was some indication from field cases that not all birds with signs of the disease died, a study was done to ascertain if the birds did recover and to find the extent of the permanent damage or degree of regeneration of liver tissue in these recovered birds. Laparotomies were performed on 25 birds which showd signs of "hepatitis" on April 30, 1955. An incision 4 cm in length was made behind the last rib and a small flashlight was inserted into the incision. By careful manipulation of the flashlight and with the use of tissue forceps the various lobes of the liver could be seen. No attempts were made to suture the

incison. Thirteen birds showing lesions indicative of "hepatitis" were moved to a small pen for further observation.

On July 15, 1955, the surviving positive birds were killed. The birds were exsanguinated and the serum was frozen for future serological studies. All organs with gross lesions were placed in 10 percent formalin for histopathological studies. Tissues from the liver, heart, and spleen, regardless of gross lesions, were also preserved in 10 percent formalin for histopathological studies. All tissues were sectioned, stained with the Mayer hematoxyalin and eosin method, and examined microscopically for lesions or evidence of repair.

Attempts at developing a serological test. Antiserum used in the following described tests (hemagglutination, complement fixation, and the conglutination tests) were attempted in both rabbits and chickens. Delaplane, *et al.*[1] reported that rabbits two to three weeks of age were susceptible to the agent. On February 9, 1955, two, three-week-old rabbits were inoculated intraperitoneally with one ml of infectious chorio-allantoic fluid. Nine days later one rabbit was moribund and was destroyed. A necropsy showed white areas two to five mm in diameter scattered diffusely throughout the liver. Inoculation of a suspension from this liver into seven-day-old chicken embryos yielded the agent in question. The other rabbit was allowed to recover and blood serum was collected June 23, 1955.

Two other rabbits were inoculated with two ml of infective chorio-allantoic fluid intraperitoneally on March 15, 1955. A week later a second injection of two ml of a 50 percent suspension of infectious chicken liver material was given. These rabbits were killed June 23, 1955, the serum collected and stored at —20 C.

Serums were also harvested from the chickens that recovered from actual field cases of the disease as described above.

Hemagglutination and hemagglutination inhibition. A hemagglutination test[3] was investigated as described for pleuropneumonia-like-organisms (PPLO). Chorio-allantoic fluid was used as an antigen.

Complement fixation test. The complement fixation test was next utilized as a means of measuring antibodies. The method as described by Hepler[4] was used for running the test. Three diferent types of antigen were utilized. Four chicken embryos, seven days old, were inoculated with the 86th passage of the original isolation of this agent. Each of three materials, yolk sac, embryo, and chorio-allantoic fluid was harvested from four eggs and processed for antigen.

Yolk sacs were harvested aseptically and washed in isotonic saline solution until all yolk material was removed. The yolk sacs were placed in a glass tissue grinder* with a clearance of 0.004 to 0.006 inches and ground until a homogeneous mixture was obtained. The emulsion of yolk sac which passed the tissue grinder was diluted with saline solution to a 20 percent suspension, frozen overnight, thawed, and strained through four thicknesses of gauze. It was stored at —20 C.

The embryos were also harvested aseptically and washed with sterile saline solution. The heads were removed and the remaining material was ground in the glass tissue grinder. The emulsified material was diluted with sterile saline solution to 20 percent suspension, frozen overnight, thawed, and strained through four thicknesses of gauze. The prepared material was stored at —20 C.

Sterile chorio-allantoic fluid from these four eggs were placed in separate containers, and was stored at —20 C. Antigens from normal embryos were prepared in the same manner as controls for all tests.

Rabbit sera were utilized in these studies because chicken serum is anti-complementary and the complement fixation inhibition test must be used in the testing of fowls. To run the complement fixation inhibition test, a source of positive non-anti-complementary serum must be available. Rabbits are susceptible to the agent and the sera are not anti-complementary.

* Corning Glass Company

Conglutination test. The procedure as described by Dimopoullos *et al.*[2] was used in these studies. This test is applicable to animals with anti-complementary substances in their blood.

Ultracentrifugation studies. These studies were carried out to ascertain the physical properties of the agent. The material for centrifugation was prepared by inoculating 30 6-day-old chicken embryos. When the embryos died, sterile chorio-allantoic fluid and yolk fluid were harvested and pooled. Approximately 100 ml from each pooled fraction was centrifuged in a Serval high-speed centrifuge at 15,000 rpm for 30 minutes. The sediments were resuspended in two ml of sterile isotonic saline solution and stored at —20 C.

The supernatant fluids were recentrifuged at 52,600 rpm for 45 minutes. Three distinct zones were seen in the chorio-allantoic substance: a flocculent, white material at the top which made up approximately one-twelfth of the volume, a sediment at the bottom of the tube, and a clear portion which made up the remainder. The two upper fractions were removed and placed in sterile containers. The sediment was resuspended in two ml of sterile isotonic saline solution. Recentrifugation of the yolk yielded a sediment but no fractions in the supernatant.

All seven fractions were inoculated into six-day-old embryos to check for the presence of virus. Three serial passages from all dead embryos were done to confirm the embryo deaths as evidence of growth of the agent.

Normal embryos were processed in the same manner as the infected ones.

Pathology. Ten selected hens which died of the disease in the field were used in this study. In each case, portions of liver, spleen, bone marrow, kidneys, and intestine were preserved in 10 percent formalin. These tissues were then dehydrated and mounted in paraffin by usual pathological techniques[6]. The tissues were sectioned approximately six microns in thickness. The sections were stained by Mayer's hematoxyalin and eosin method[6]. Each tissue was studied microscopically to determine the lesions, if any.

The same tissues as above were saved from three normal hens from a non-susceptible strain of birds. These tissue sections served as controls for comparative purposes.

RESULTS

Immunity studies. The results of inoculating birds on farm A (Group I) with one injection is shown in table I and figure 1. Mortality from hepatitis in the inoculated group is about one-half of that of the controls (table I); whereas, mortality in birds dying from other causes is comparable in the two groups. There was no significant difference in the growth rate as measured by average weight in the two groups. The peak of mortality as a result of "hepatitis" occurs approximately one month after the peak of egg production (figure 1). One inoculation would appear to reduce mortality due to "hepatitis" in the early laying period. Of the 20 control birds

TABLE I

Number of birds with hepatitis and other lesions following attempted immunization

Farms and numbers of chickens		Hepatitis	No lesions	Reproductive disorders	Hjarre's disease	Cannibalism	Leucosis	Misc.
Farm A	Group I							
	1000 inoculates	56	53	16	19	12	7	2
	1000 controls	100	58	15	20	7	3	2
	Group II							
	1000 inoculates	5	37	23	2	27	1	19
	1000 controls	5	39	10	2	52	1	18
Farm B	Group III* 2 wks.							
	1000 inoculates	2						
	1000 controls	32						
	Group IV* 5 wks.							
	500 inoculates	50						
	500 controls	51						
	Group V* 8 wks.							
	500 inoculates	37						
	500 controls	66						

* Approximately 5 months after the first inoculation Groups III, IV, and V received a second inoculation and only hepatitis mortality records were kept on these groups.

used to determine infectivity of the inoculum and killed 10 days after inoculation, 19 had typical heart and liver lesions.

In the 1955 studies on farm A, there was only a limited number of hepatitis cases in either the inoculated or control (table I) groups. No explanation could be made for the lack of hepatitis in either the controls or inoculates. The 100 male chicks used to determine infectivity of the inoculum were killed at 10 days of age. Of these, 43 had liver and 50 had heart lesions.

The two older groups on farm B (Groups IV and V) were in egg production when the inoculation was repeated. Egg production in Group V was at approximately 55 percent and Group IV 15 percent. In both of these groups, egg production was decidedly retarded. Group III had a low incidence of hepatitis throughout the period of observation.

TABLE II

Average weight of group I birds for one month

	Average weights	
	Controls	Inoculates
March 25, 1954	47.8 gm.	47.8 gm.
April 1, 1954	79.3 gm.	76.2 gm.
April 8, 1954	114.8 gm.	129.5 gm.
April 30, 1954	289.1 gm.	300.5 gm.

Studies of recovered birds. Of the 13 birds which had lesions of hepatitis on laparotomy, two died soon after the operation due to rupture of the liver. Two birds died two days later and had typical lesions of "hepatitis". One bird regained body weight, started laying and apparently had recovered. After laying for one week, it suddenly died with typical lesions of "hepatitis". The agent was isolated from this bird by inoculating chicken embryos. Eight of these birds apparently recovered and were later killed and examined. Necropsy findings are as follows.

Hen 491497 had been laying about one week before she was killed. Grossly the liver had an area of dark brown material one-half cm in diameter on the surface located near the center of the left lobe. Microscopically this area was an old hemorrhage. There was a proliferation of reticulo-endothelial

cells around and considerable hemosiderin in and around it. There were numerous multinucleated giant cells and eosinophils around the periphery of the hemorrhage.

Hen 491500 had numerous ova many of which were almost mature. There was an area of dark brown material from three to four mm in diameter adhering to the edge of the right lobe of the liver. Microscopically no lesions were apparent in the liver.

Hen 490567 had been laying for two weeks before death. The bird was very fat and the liver was pale yellow. Several small scars were located on the surface of the right lobe of the liver. Microscopically no lesions were apparent in the liver.

Hen 489748 did not have any mature ova when killed. There were numerous scars on the surface of the liver. Microscopically there was a hyperplasia of the epithelium of the bile ducts. The liver parenchyma had no evident lesions.

Hen 490371 had been laying for approximately two weeks before she was killed. There was a yellowish, fibrin-like area three to four mm in diameter adhering to the right lobe of the liver. Microscopically no lesions were apparent in the liver. The yellowish nodule was lost in the processing of the tissue.

Hen 491494 was not laying at the time of death. There were no visible gross or microscopic lesions.

Hen 489769 was not laying. No gross nor microscopic lesions were observed.

Hen 490969 was not laying at the time of death. The right lobe of the liver had a scar approximately two cm in diameter. Microscopically no other lesions were apparent in the liver.

Attempts at developing a serological test. None of the serological tests attempted revealed any measurably antibody with the technics used. It was noted previously that rabbits had a natural complement fixing antibody against yolk sac antigen.

Ultracentrifugation. Table III indicates that the agent was concentrated in the turbid material at the top of the

TABLE III

Mortality of six-day chicken embryos inoculated with fractions of ultracentrifuged material in trial 1.

No. passage	I/C	Date	Inoculum	No. of deaths on each post-inoculation day 1	2	3	4
105 Liv.	5/5	11-19-55	Yolk sed. 52,640 rpm	0	2	0	0
105 Liv.	5/5	11-19-55	Yolk sed. 15,000 rpm	1	0	0	0
*2nd pass.	5/5	11-26-55	From egg I above	0	0	0	0
105 Liv.	5/5	11-19-55	Yolk supernatant	0	0	0	0
105 Liv.	5/5	11-19-55	CA fluid 52,640 rpm	0	0	0	0
105 Liv.	5/5	11-19-55	CA fluid 15,000 rpm	0	0	0	0
105 Liv.	5/5	11-19-55	CA fluid supernatant	0	0	0	0
105 Liv.	5/5	11-19-55	Supernatant materials after 52,640 rpm	0	2	2	1
2 M T**	5/5	11-26-55	Supernatant material after centrifugation of second passage	0	0	3	2
3 M T**	5/5	11-26-55	3rd passage	1	0	3	1

NOTE—No embryos died after the 4th day

* Second passage from egg that died from 1 above

** MT = supernatant after 52,640 rpm centrifugation of the second and third passage respectively

† Various other fractions as mentioned gave negative results and are not shown in the table

fluid when centrifuged at 52,640 rpm. This material was the only one of the seven fractions which killed six-day-old chicken embryos in a characteristic pattern typical of hepatitis. A repetition of the experiment gave results comparable with trial I.

Pathology. Focal areas of acute inflammation were found in the kidneys and heart.

In approximately 15 percent of the cases that died, a distinct enteritis occurred. The intestinal contents were reddish-brown and very fluid in consistency. Microscopically, the epithelium was either denuded or necrotic and a large area had disappeared from the villi. In some cases the only recognizable part of the villus was a small strand of connective tissue. The lumen of the intestine was filled with necrotic debris, red blood cells and polymorphonuclear leucocytes.

The bone marrow of affected birds had a high percentage of large myelocytes as compared to normal chicken bone marrow. There was almost a complete absence of the mature type of granulocyte in the affected birds.

There were areas of necrosis scattered throughout the liver. These areas on gross observation, were white foci varying from five mm to four cm. The lymphatic nodules appeared to be larger and more numerous in affected as compared to normal birds. In some cases, the liver contained cavities filled with blood. Immediately surrounding the necrotic areas were zones of inflammation composed of reticulo-endothelial cells and a varying number of eosinophils. The areas of necrosis have no constant position in the liver lobule. In some cases the areas of necrosis were so extensive that several lobules were involved. Rupture of the capsule and fatal hemorrhage also occurred.

Pathologically the disease is similar to virus hepatitis in man in that acute degenerative changes accompanied by an acute inflammatory process exists.[9]

The spleens of the affected birds had a decided lack of lymphopoietic activity. In some cases, only a very few lymphocytes surround the central artery, while in severe cases the spleen is practically devoid of lymphocytes.

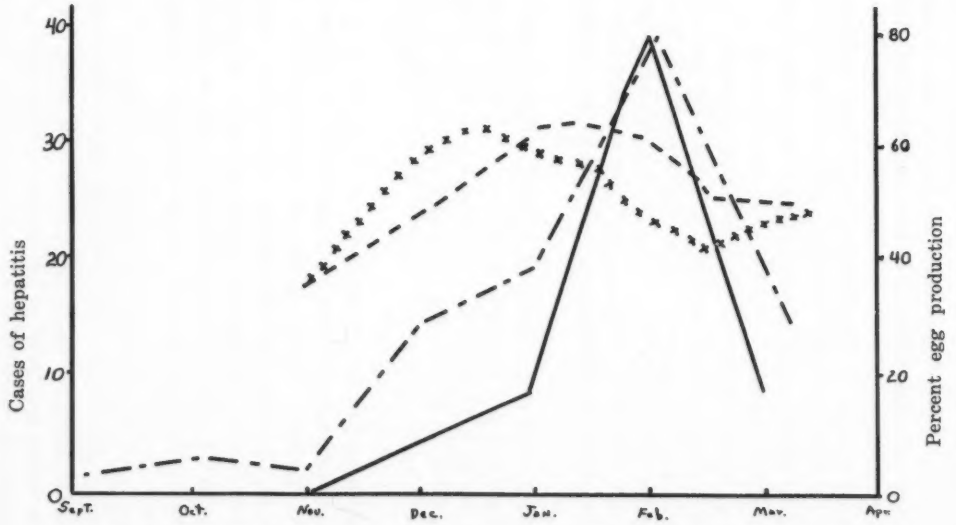

Fig. 1. Hepatitis and egg production of inoculated and control birds by months in group 1.

Inoculates — Egg production — Hepatitis mortality
Controls × × × × × × × × — · — · — · —

DISCUSSION

The lack of disease in a second group of farm A (Group II) is unexplainable. There was a reduction in egg production in the control lot as compared to the inoculates but this apparently is not significant.

Birds that were in production when the second injection was given (Groups IV and V) had a decided increase in the incidence of "hepatitis", indicating that this is not a desirable time for inoculation.

Apparently the morbidity is higher in this disease than mortality indicates by a continued gradual drop in egg production (Graph I). Probably a large number of the birds affected recover from the disease. The results of the laparatomies performed would indicate some birds recover sufficiently so that the liver is normal even on microscopic examination.

There was considerable swelling and extensive necrosis of the parenchyma of affected livers. Wherever necrosis was extensive, the necrotic areas filled with blood and were recognizable as small brownish nodules. Probably if more time had elapsed before these hens were killed these lesions might have disappeared.

The inability to detect any antibody was consistent with results obtained with the attempted immunization of the birds with the agent. Several factors could account for failure of all of these tests, such as; (1) lack of a suitable concentrated antigen (this probably has some effect) because of the low titer in chicken embryos; (2) lack of a known immune serum with a sufficiently high titer; or (3) the inability of the agent to stimulate any antibodies.

The ultracentrifugation studies partially explained the many dififculties encountered in working with this agent. The fact that the agent rose to the top of the centrifuged material was entirely unexpected. All agents studied by ultracentrifugation to date have been concentrated at the bottom of the tube. Since this agent rose to the top, it possibly indicates that it may be composed of considerable lipoprotein.

The possibility of the agent being lipid in nature could very well explain the lack of success in developing a serological test. Lipoid materials in general, are not very satisfactory antigens.[8,10] It is possible that the agent either does not stimulate measurable antibodies or the lipoid substance interferes with the antigenic action of the agent. If the substance is a lipid this fact would explain the short life of the agent in storage. The oxidation of the lipid material could very easily denature the agent in some manner rendering it no longer infective.

The primary lesion caused by this agent is located in the liver. The lymphoid exhaustion of the spleen and the rather immature type of cell in the bone marrow are probably due to the reaction to the infectious agent and its toxins. Death probably occurs due to the extensive damage of the liver.

ACKNOWLEDGMENTS

The author wishes to acknowledge the guidance and suggestions made by Dr. J. P. Delaplane and Dr. Hilton A. Smith of the Schol of Veterinary Medicine, Texas A and M College, in carrying out these studies.

SUMMARY

An agent causing hepatitis in chickens was studied to determine characteristics of that agent. Studies of birds inoculated with the agent would indicate little or no immunity developed to this agent. The inability to demonstrate any measurable antibody by any of the serological tests studied would also indicate little or no immunity. Study of livers from known recovered birds indicate that some birds recovered with complete return to normal of the diseased livers.

Limited ultracentrifugation studies indicated that the agent was not sedimented but rose to the top of the centrifuge tube. Microscopic lesions consisted of severe necrosis of liver parenchyma with an infiltration of leucocytes. The spleen showed lymphoid exhaustion. In some cases, severe necrosis of the intestinal mucosa was noted.

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INDIRECT TRANSMISSION OF INFECTIOUS LARYNGOTRACHEITIS IN CHICKENS

By F. W. KINGSBURY and E. L. JUNGHERR

Department of Animal Diseases
University of Connecticut, Storrs, Connecticut

Received 18 July 1957

THE laboratory of the Department of Animal Diseases diagnosed 15 cases of infectious laryngotracheitis (IL) from the same community between May 6 and August 15, 1955. Two more cases were diagnosed in the field. By ob-

taining a detailed history from each poultry farm known to be affected, the mode of transmission between cases was ascertained and evaluated in the light of preventive sanitation and recommendations for vaccination.

The theory of direct transmission (carrier bird to susceptible bird) of the IL virus has been accepted since Gibbs' studies in 1931. However, direct transmission between cases in this Connecticut community was unconfirmed. Evidence substantiated several means of indirect transmission which have been suggested in previous publications. In 1931, Gibbs stated that controlled experiments demonstrated that "man may serve as a mechanical carrier of the virus when excretions are fresh and carried directly from sick to healthy susceptible birds". Hofstad (1952) granted that "transmission of the disease indirectly by way of contaminated poultry crates, other equipment and by flying birds would be possible, although it is of minor importance in the spread of laryngotracheitis". The 1955 Report of the Committee on Morbidity and Mortality stated that the virus may be carried "on the hands and clothing of humans (neighbors, salesmen, vaccinating crews, blood testers. itinerant poultry buyers, etc.) or on infected poultry equipment of any kind . . . prevention of the disease requires only the avoidance of the practices which enable the disease to reach the healthy flock".

The Connecticut cases were apparently spread by such mechanical carriers as men, flying birds (crows), rats, and dogs. An educational program, to inform poltrymen affected by these outbreaks, was undertaken while interviewing them with the help of the County Poultry Agent and of the U.S. Fish and Wildlife Service Agent who emphasized rodent control.

CASE HISTORIES

The geographic and chronologic interrelationship of the various outbreaks is shown in figure 1 and table I.

The first appearance of IL followed capetting on two broiler farms by a crew that had contacts with another infected area in a neighboring county. This capetting crew visited farm 1 on April 30, apparently transmitting IL, which

was diagnosed at the laboratory on May 6. About 3400 birds died; the carcasses were buried. No vaccinating was done, as the broilers were sold and the coops remained idle for approximately two months.

The birds on farm 2 were capetted on May 1 and 2 and contracted IL about May 10. Mortality reached 3,200 birds. The disposal pit was filled before the outbreak terminated. Thereafter, disposal methods became a matter of concern, due to the central location of farm 2 among subsequent cases. The one mile stretch surrounding farm 2 was popularly tagged "Laryngo Row". The IL status of farms 2 and 3 was discovered while interviewing neighbors with affected flocks.

On farm 3 the owner denied knowing about the existence of the diagnostic laboratory. Transmission followed the transfer of broilers to range shelters with the help of neighborhood boys. There were 1500 birds that died and disposal methods were open to question. Only 4 days were taken for clean-up after depopulation, but the incoming lot was vaccinated.

Rat activity was prominent throughout the "Laryngo Row" neighborhood, with one exception. Farm 4 was the most sanitary farm affected in the community. On June 1, IL was diagnosed in the sex-linked cross layers. Transmission was attributed to a White Rock carcass, probably from farm 3 where mortality was high at the time, which was brought home by the dog a few days before. An early diagnosis was made, all birds were vacccinated, and mortality was approximately 100 of the 3500 birds. The owner of farm 4 did not want his neighbors to know about his outbreak.

The outbreak of IL at farm 6 followed a visit of the poultryman from farm 4, who entered his friend's coops to examine some recently constructed equipment. Diagnosis was made on June 10 and 1800 layers were vaccinated. The owner did not examine for "takes". About 250 birds died and were burned, then buried. Approved warfarin bait stations were used for rat control as on farm 4. Farms 2, 3, 5, and 7 had many signs of rat infestation on their premises. Control

TABLE I.

Chronologic interrelationship of infectious laryngotracheitis outbreaks

Farm case number	Date of diagnosis, 1955	Most probable means of transmission	Number of birds		Mortality %
			Meat	Layers	
1	May 6	Capetting crew, April 30	17500		20
2	May 10 June 16	Capetting crew, May 1 and May 2	15100		21
3	May 15 June 16	Boys from neighborhood helped to move birds	14000		11
4	June 1	Dog brought home dead chicken week before		3500	3
5	June 6	Neighbor to Farm No. 2 on "Laryngo Row"—rats	6000	4000, 2000	25, 20, 0
6	June 10	Owner of Farm No. 4 visited coop	3600	1800	14
7	June 15	Rats, also on "Laryngo Row"	8700		7
8	June 20	Crows and rats	4000		30
9	June 24	Crows with range pullets	4000	2200	9, 7
10	June 27	Town dump area	2200		23
10A	—	Home farm	8000	2500	
11	July 12	Town dump area	2500	3500	.5
12	July 12	On "Laryngo Row"	4000	3300, 2000	7, 21, 10
13	July 13	"Laryngo Row" extension	5500	6500	25
14	July 21	Neighbor to Farm No. 10A	7500		4
15	July 28	Uses same rat infested disposal area as Farm No. 10A	4000	3300	.5
16	Aug. 15	½ mile from town dupm	8000	4000	2

measures in this neighborhood were haphazard, if any. One man, the owner of farm 2, stated that he rolled warfarin into papers which he threw into corners!

On June 6, IL was diagnosed at farm 5. About 3000 sex-linked cross breds were vaccinated when sick with a loss of 1500. The 3000 more that were vaccinated while healthy survived without loss. Approximately 4000 layers and 2000 pullets were also vaccinated with a loss of 800 birds. The disposal pit was full and rat signs were prominent.

Specimens from farm 7 came to the laboratory on June 15 and IL was diagnosed. The following day 8700 birds were vaccinated. About 600 birds died of IL. A wood-burning stone incinerator was used for disposal. However, dead birds, fresh rat burrows, and grain in a bag were observed in a range shelter. Trash, manure and weeds surrounded the coops.

There were 4000 broilers housed in 3200 square feet of floor space on farm 8 where rats were also present. The birds were sick for some time before diagnosis was made on June 20. The disease had gone through two coops, but had not appeared in a remodelled barn. Vaccinating on farm 8 was done in the barn only. Mortality was 1200. Dead birds were thrown into pig pens where many crows visited. Crows were observed on the poultry range of the neighbor to the east. This poultryman vaccinated his birds when he was informed of IL on farm 8. Birds were examined for "takes" and the flock remained normal.

Transmission to farm 9, where IL was diagnosed June 24 and which was located two miles from farm 8, was attributed to crows. Among the 2000 range pullets, 150 birds were lost. Both hatching eggs and broilers were produced on farm 9. Only layers were vaccinated. Later the 4000 broilers had an outbreak of IL with a loss of 350 birds. Warfarin bait stations were used on this farm. Dead birds were buried.

The IL outbreaks on farms 10, 11, and 16 could be attributed to their proximity to the town dump which was found to be heaped with carcasses, too numerous for any normal flock mortality. However, these farms had also been visited by itinerant poultry buyers previous to the IL out-

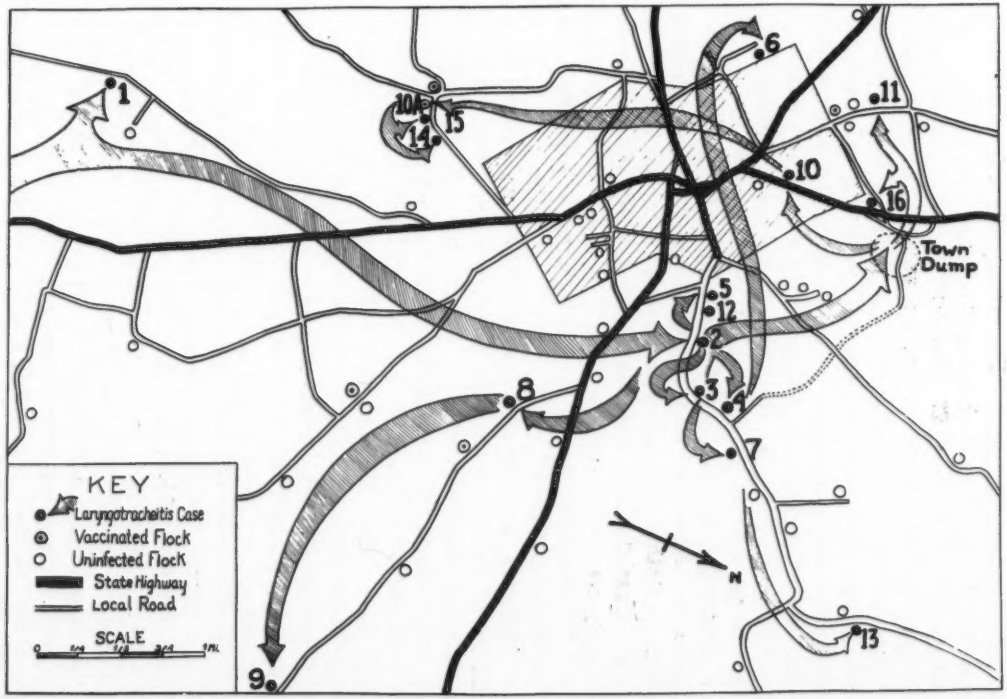

Fig. 1. Probable progress of disease. Numbers refer to outbreaks listed in table 1.

breaks on them. On farm 10 the owner related his flock's IL outbreak to the sale of his capettes two weeks before IL was diagnosed on June 27. The disease had made so much headway before diagnosis that no vaccination was undertaken. Of the 2200 sex-linked cross pullets, 500 died. This same poultryman travelled daily between farm 10 and his home farm, 10A, where he had 2,500 two-year-old layers and 8000 broilers. Carcasses from farm 10 were dumped into a gravel pit 200 feet from the coops at farm 10A. There were signs of rat activity throughout this neighborhood.

Farm 11 where IL was diagnosed July 12 had had many visitors to the coops. One of the two coops of layers was affected. Both coops were vaccinated. The coop of broilers was uninfected and subsequently went unvaccinated. Due to an early diagnosis, only 15 birds died. The poultryman on farm 11 practiced no rat control program. Carcasses were burned in an oil drum.

Farm 12, another on "Laryngo Row", was owned by a relative of the poultryman at farm 5. There was a distance of 200 feet between their coops. Diagnosis of IL was made on July 12. Losses included 300 of 4000 broilers, 200 of 2000 pullets, and 700 of the 3300 layers. All birds were vaccinated after the outbreak was diagnosed. Succeeding lots of broilers were not vaccinated. A stone incinerator, used for disposal, became heaped with carcasses. Warfarin (without bait stations) was occasionally used, as rats had been seen on the premises.

Farm 13 where IL was diagnosed on July 12 was located beyond farm 7. Only 13-week-old capettes were affected. Of 6,500 birds, 1,600 died. The 5,400 pullets were all vaccinated, but not checked for "takes". Dead birds were supposedly burned in the oil drum, yet neighbors reported piles of carcasses in the woods behind the coops. The poultryman on farm 13 planned to vaccinate pullets but not the broilers.

Farm 14, near farm 10A, had IL diagnosed on July 21. Just the day before, the owner of farm 10A had sold his two year-old layers and vaccinated his five and seven-week-old broilers. He denied having IL on his farm, but vaccinated

just the same. His neighbor to the west vaccinated when advised of the diagnosis at farms 14 and 15 and had no disease outbreak. At farm 14 there was a loss of 300 of the 7,500 broilers. Birds were depopulated without vaccinating. Carcasses were buried and new birds were to be vaccinated. Farm 15 where IL was diagnosed a week later on July 28, did vaccinate with a loss of only 15 birds. Neither farm had an approved rat control program. Both claimed they buried dead birds.

Farm 16 where IL was diagnosed on August 15 was owned by a poultry dealer, the father of the poultryman at farm 12. Of 8,000 broilers, 150 died. The 4,000 layers were vaccinated. Although located across from the dump, warfarin was used only occasionally. Dead birds were buried.

EDUCATIONAL MEASURES

All poultrymen in this area were notified of the outbreak by a letter from the County Poultry Agent on June 17. The letter requested them to "be especially careful about the immediate disposal of dead birds by deep burying or complete burning". A series of similar letters kept poultrymen informed of the recommendations of the Department of Animal Diseases and the course of this enzootic disease.

The Extension Veterinarian in Poultry Diseases, accompanied by the County Poultry Agent, visited each farm to advise and recommend measures applicable to each case. Emphasis was given to:

1. Emergency and preventive vaccination (including checking for "takes").
2. Disinfecting clothing and equipment before contact with healthy birds.
3. Disposing of dead birds immediately by burning or burying.
4. Exterminating rats by means of warfarin bait stations. This last point was further discussed by the U. S. Fish and Wildlife Service Agent on the second visit, made to determine the extent of the rodent problem on the affected farms.

The County Poultry Health meetings in October were designed to inform poultrymen throughout the State about the means of indirect transmission of IL as well as the above recommendations.

RESULTS

Eleven of the 16 poultrymen vaccinated their birds, but only the two owners of farms 9 and 11 examined their vaccinated birds for "takes". There were just two farms, 11 and 15, where an early diagnosis was combined with immediate emergency vaccination to prevent high flock mortality. The follow-up survey made five months later showed that seven poultrymen who produced both meat and eggs had been raising their broilers without preventive vaccination after the outbreak.

Among the 15 poultrymen interviewed on the follow-up survey, the four on farms 2, 5, 8, and 12 admitted that they needed more adequate disposal methods during the outbreak and were still contemplating the purchase of commercial incinerators. The others felt no need for improvement. Nothing concrete had been accomplished in this direction at that date.

Only three poultrymen were using approved rodent control methods (warfarin bait stations), five months after the outbreak. Two thought they had no rats and used no control methods, while nine used warfarin haphazardly and irregularly. One poultryman used no rodent control although he knew the area was rat infested. The town dump was still being used for dead birds in large numbers. Refuse was neither burned nor buried.

SUMMARY

Transmission of infectious laryngotracheitis (IL) to five farms, 1, 2, 3, 6, and 10, was attributed to human beings and their carelessness in disinfecting clothing. At farm 4, infection followed a dog retrieving a carcass. At another farm, 9, IL was apparently brought to a range flock by crows from a piggery, where IL carcasses were dumped. At the other eight farms, IL was ascribed to a combination of poor disposal methods (including carcasses at the town dump, in the woods, and piled on incinerators) and prominent rat activity.

Five months later, conditions on most farms remained unimproved and few of the poultrymen affected by this enzootic disease had followed recommendations despite their combined loss of more than $18,000 in mortality alone.

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THE EFFECT OF ESTRADIOLCYCLOPENTYLPROPRIONATE (ECP) ON THE BLOOD CELLS OF CHICKENS

By CHARLES D. DIESEM, J. K. BLETNER and WALTER G. VENZKE

College of Veterinary Medicine
Ohio State University, Columbus, Ohio

Received 19 Sept. 1957

ESTRADIOL cyclopentylproprionate was administered parenterally to a group of chickens to determine the effect of the hormone on the peripheral blood hemopoietic tissues. Conner[3] reported that repeated doses of estrogens in dogs produced a leukocytosis, oligocythemia, oligochromemia, thrombocytopenia and agranulocytosis. These conditions were followed by a leukopenia. Osgood[7] and Wintrobe[13] have reported drugs such as arsenicals, benzene, dinitrophenol,

and the sulfonamides are capable of producing hypoplastic anemia in man. Rigdon[8] has shown that sodium selenite will inhibit the maturation of duck erythrocytes. He also demonstrated that this inhibition of erythrocyte production could be reversed by using thiols. Shapiro[10] reported that crude adrenal cortical extract produced absolute lymphopenia, a decrease in erythrocytes and an absolute polymorphonuclear leukocytosis. Bannister[2] injected cortisone acetate daily for 13 days into chickens and found the results paralleled the lymphopenia and eosinopenia observed in mammals. Huble[6] noted that chickens treated with cortisone acetate and ACTH showed a lymphopenia, an increase of heterophils, a leukocytosis and an 80 percent decrease in eosinophils. The eosinopenia observed was not in agreement with the findings of Stamler.[11] Siderius[12] found dihydroergotamine influences the physiological effect of adrenalin by preventing a lymphopenia.

MATERIALS AND METHODS

Twenty-four eight and one-half week old Single Comb Leghorn cockerels and pullets were separated into equal numbers of each and were placed in two batteries. The diet fed one group of twelve chickens was a 22 percent protein broiler ration. The second group received the same broiler ration except it was supplemented with 0.1 percent sulfaquinoxaline. The sulfaquinoxaline was fed at a high level in order to determine if it might interfere with the normal hemopoietic activity of young chickens. The drug was added to the feed five and one-half weeks prior to starting the blood counts or when the chicks were three weeks of age.

Eight chickens from each battery of twelve birds were given ECP while the other four birds were used as non-injected controls. The synthetic hormone injected was estradiol cyclopentylproprionate (ECP). The hormone was administered in doses considered to be in excess of therapeutic levels in order to insure its toxic potential in chickens. Eight chickens in each battery received a total of 12 milligrams of the hormone during a 5-day test period. This dosage divided into four individual injections was administered parenterally

TABLE I

The effect of ECP on the hemograms of chickens fed 22% protein ration

Group	Total RBC	Total WBC	Baso-phils	Myelo-cytes	Hetero-phils	Lympho-cytes	Mono-cytes	Date
Control (746)	3,172,250	23,250	543	543	3,344	17,621	1,198	4/2
Injected (740)	3,210,000	15,000	375	87	3,852	10,401	283	4/2
Control (742)	3,145,000	22,750	1,138	182	3,972	15,477	1,978	4/3
Injected (755)	3,170,000	18,375	9,600	30	7,349	9,270	525	4/3
Control (760)	3,290,000	17,875	810	0	4,612	12,196	256	4/4
Injected (785)	3,253,750	20,375	505	82	5,294	14,198	293	4/4
Control (764)	3,117,500	18,375	450	0	5,793	11,183	240	4/5
Injected (815)	2,810,000	22,000	551	141	3,261	17,721	323	4/5
Control (785)	3,217,500	15,250	423	66	1,270	13,073	0	4/6
Injected (836)	2,631,250	17,125	203	149	2,844	13,856	71	4/6

( )—Numbers in parenthesis indicate average weight of birds in grams.

into the pectoral muscles. The ECP utilized contained 2 mg per ml of the active hormone. The first and second series of injections consisted of 1 ml of ECP. The third and fourth injections consisted of 2 ml of ECP or four milligrams of the synthetic hormone.

Prior to each injection of the hormone blood samples were taken from the radial or ulnar veins of the birds and blood counts were made. Thrombocyte counts were made every other day and body weights were determined daily. The technique used for making the thrombocyte counts was that described by Dennington and Lucas.[4] At the termination of the experiment a necropsy was performed on each bird and a histopathological examination was made of the genital organs. Weights of adrenals and gonads were recorded.

RESULTS

Table 1 shows the hemogram averages on control and ECP injected chickens fed a standard broiler ration. Table 2 shows the hemogram averages for control and ECP injected birds fed a standard broiler ration supplemented with 0.1 percent sulfaquinoxaline. The results of the study indicate that the only effect the hormone had on the complete blood counts was to reduce the total erythrocyte counts. This reduction in erythrocytes was not associated with any clinical signs of anemia. Thrombocyte counts did not show any changes that were statistically significant (table 3).

Necropsy findings revealed no gross changes in any organs other than those of the genital tract of the females. The injected pullets, although only nine and one-half weeks of age, when necropsied, had oviducts that were approximately one centimeter in diameter. The gross size of the magnum area of the oviduct in the injected birds grossly resembled a similar area of the oviduct of a non-laying hen. The oviducts of young non-injected pullets were difficult to find by dissection of the genital tracts. Their walls were thin and the oviduct was only one to two millimeters in diameter. This observation indicated that the hormone did have an effect on oviduct tissue, but the hormone did not produce any gross or histological alterations in the gonads of pullets or cockerels during

TABLE II

The effect of ECP on the hemograms of chickens fed 0.1% sulfaquinoxaline plus 22% protein ration

Group	Total RBC	Total WBC	Baso-phils	Myelo-cytes	Hetero-phils	Lympho-cytes	Mono-cytes	Date
Control (598)	3,412,000	17,750	417	945	4,642	10,785	960	4/2
Injected (700)	2,900,000	16,687	236	241	1,760	11,305	1,695	4/2
Control (593)	3,265,000	13,825	468	0	8,167	6,678	560	4/3
Injected (724)	2,898,750	18,687	460	0	3,536	12,938	922	4/3
Control (632)	3,100,000	14,000	680	32	1,360	14,960	1,031	4/4
Injected (754)	2,793,000	21,937	321	55	5,699	15,605	256	4/4
Control (629)	3,025,000	16,125	183	281	2,996	12,345	318	4/5
Injected (773)	2,745,375	26,625	276	30	5,670	20,420	221	4/5
Control (643)	3,305,000	13,500	207	1,507	2,115	9,527	142	4/6
Injected (767)	2,682,000	20,888	301	200	4,977	15,125	172	4/6

TABLE III

The effect of estrogen (ECP) on the thrombocyte count of chickens and genital organs

Bird number and ECP dosage	Date of count 4/2	4/4	4/6	Gross observation of genital organs
	Chickens received sulfaquinoxaline .1% in feed			
8707 (none)	22,888	13,416	51,354	Oviduct diameter 0.1 cm
8712 (none)	46,296	28,796	42,240	Oviduct diameter 0.1 cm
8717 (none)	10,070	14,124	23,730	Genitalia normal (M)
8720 (none)	42,036	43,400	26,880	Genitalia normal (M)
8703 (12 mg)	40,788	44,156	30,938	Genitalia normal (M)
8704 (12 mg)	36,580	38,640	49,560	Genitalia normal (M)
8705 (12 mg)	39,480	22,320	64,272	Oviduct congested. Diam. 0.7 cm
8708 (12 mg)	20,180	20,506	44,180	Genitalia normal (M)
8711 (12 mg)	8,316	35,222	48,780	Oviduct diameter 1 cm
8713 (12 mg)	28,476	25,213	45,500	Oviduct diameter .8 cm Congested
8718 (12 mg)	39,680	26,100	38,734	Genitalia normal (M)
8719 (12 mg)	28,930	61,812	61,608	Genitalia normal (M)

TABLE III—(Continued)

		Chickens received no sulfaquinoxaline in feed		
8728 (none)	43,884	26,226	46,376	Oviduct diameter under 0.1 cm
8730 (none)	29,340	42,588	35,360	Oviduct diameter under 0.1 cm
8731 (none)	22,250	73,680	26,680	Normal genitalia (M)
8736 (none)	19,720	54,462	69,360	Normal genitalia (M)
8721 (12 mg)	38,874	22,866	51,240	Normal genitalia
8722 (12 mg)	19,224	37,990	48,260	Normal genitalia (M)
8723 (12 mg)	63,664	23,934	63,928	Oviduct diameter 0.8 cm—congested
8725 (12 mg)	27,880	63,856	34,300	Oviduct diameter 1 cm—congested
8727 (12 mg)	26,048	56,712	91,006	Normal genitalia (M)
8732 (12 mg)	38,192	29,832	34,272	Oviduct 0.8 cm—congested
8734 (12 mg)	28,244	21,840	39,200	Oviduct diameter 1 cm—congested
8738 (12 mg)	54,448	20,944	50,350	Normal genitalia (M)

(M) = Male (12 mg) = dose of ECP given

the test period. The variation in testicular size in some males, which was noted in the more rapidly maturing birds, was not believed due to ECP since it was observed in both the controls as well as treated birds. Average weights for the gonads and adrenals did not vary significantly between controls and injected birds.

Histologic sections made from the gonads of the birds did not show conclusively that hyperplasia, hypoplasia or degeneration of the epithelium of the seminiferous tubules occurred. Figure 1 shows a histologic section of testis from an eleven week old non-injected cockerel. Figure 2 shows an area exhibiting an increased amount of interstitial tissue between sections of seminiferous tubules in an injected cockerel and some degeneration of tubular epithelium. The authors believe this to be normal variation between different areas of the testicle and not due to the effect of the injected hormone. The oviducts in the injected females furnished definite proof that the hormone has an effect on certain genital areas. Figures 3, 4 and 5 show sections of the magnum area of the oviduct from a non-laying hen, a nine and one-half week old ECP injected pullet and a thirteen week old non-injected pullet, respectively. The mucosa, lining the oviduct of the hen, is considered to be columnar epithelium. The mucosal cells are uniform in size and they usually appear lighter than cells in the submucosal layers.

Figure 4 shows the glandular tissue was stimulated in the injected pullet's oviduct and the overall size of the oviduct was increased. Figure 5 shows the epithelium was not well developed in the non-injected pullet. A cross section of the duct was so small that the entire cross section is almost shown in the field on the photomicrograph. The vascularity and congestion of oviducts of the injected birds were markedly increased while the oviducts from control birds did not show any increase in size or blood vessels or increased numbers of blood cells in histologic sections. The increased thickness of the oviduct wall of injected birds was produced by the rapid development of the submucosal glandular tissue.

No subperiosteal or intramuscular hemorrhages were

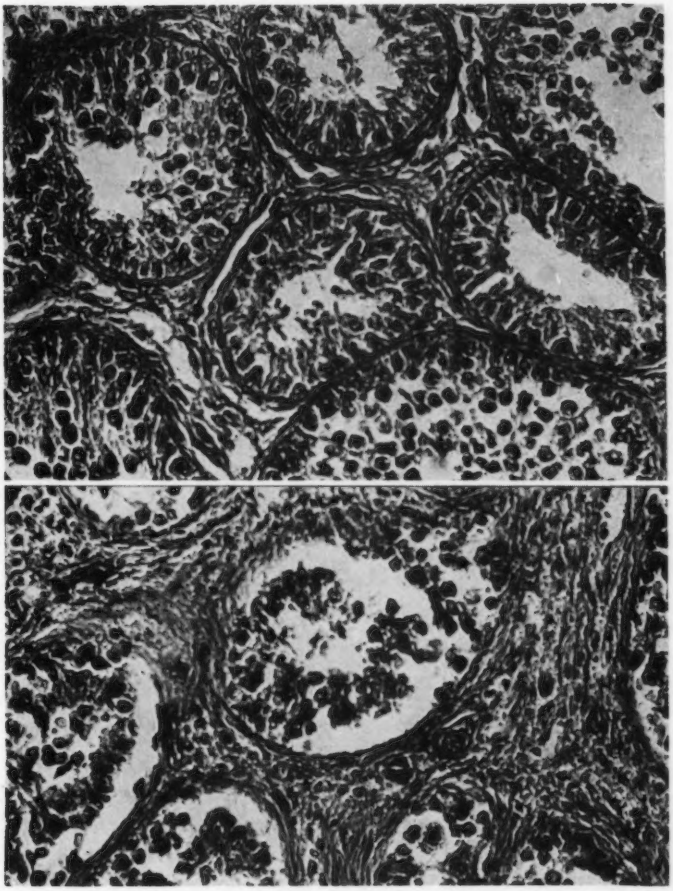

Fig. 1. (top) A section from the testis of a nine and one-half old White Leghorn male that did not receive estradiol cyclopentylproprionate. The interstitial epithelium is not extensive and the epithelium of the seminiferous tubules is intact. (Masson trichrome strain, 400×.)

Fig. 2. (bottom) A section of testis from a nine and one-half old White Leghorn male. The bird had received twelve milligrams of ECP. The interstitial tissue appears thicker in the area shown than in figure 1. All microscopic fields observed did not show this extensive interstitial tissue. The epithelium appears to be separating from the basement membrane. (Mallory trichrome stain, 400×.)

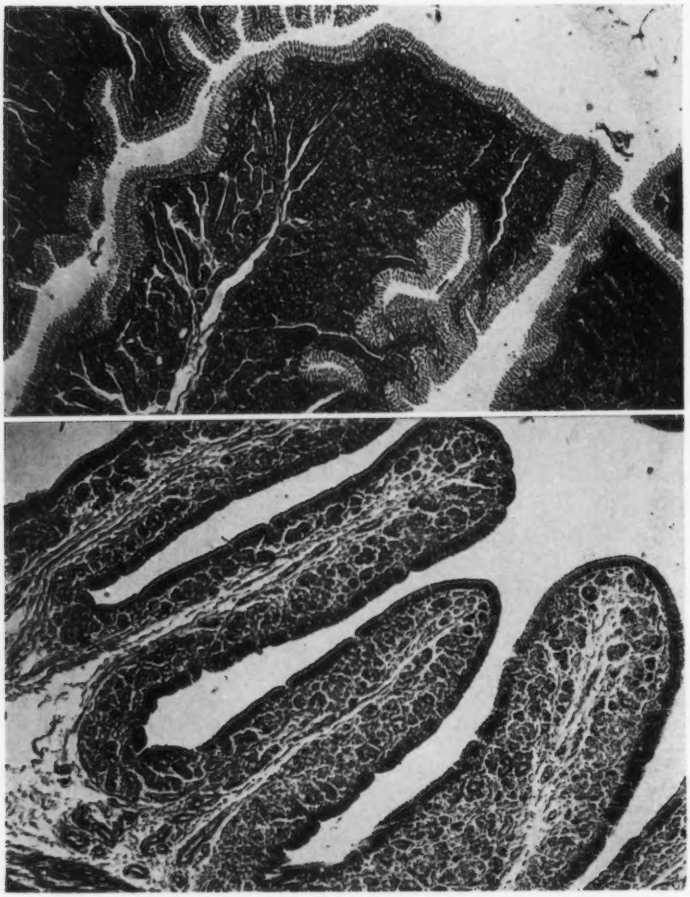

Fig. 3. (top) The magnum area of the oviduct of an adult non laying White Rock Hen. The chicken was eighteen months of age. Note the well developed glandular area of the oviduct. Masson trichrome stain, 90×.)

Fig. 4. (bottom) The oviduct of a nine and one-half week old pullet which had received twelve milligrams of estradiol cyclopentylproprionate. Note the development of the villae and the grandular area in comparison to figure 3. (Masson trichrome stain, 90×.)

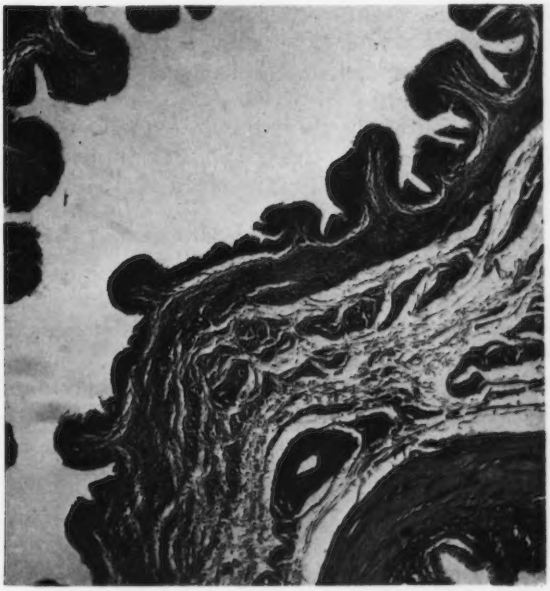

Fig. 5. The oviduct of a thirteen week old pullet showing the magnum area occupying the upper portion of the field. The glandular area underlying the mucosa has not developed. The tissue was taken from a non-injected control. (Mallory trichrome stain, 90×.)

noted on necropsy. Bone marrow smears from injected birds were no different in appearance than smears taken from all control birds. Accessory splenic tissue was observed in the area of the junction between the proventriculus and the gizzard.

DISCUSSION

The results in the preceding section indicate that an estrogenic hormone such as estradiol cyclopentylproprionate has no marked effect on the cellular content of the blood. The marrow of the chicken injected with the hormone was not inhibited from functioning as a hemopoietic organ.

The birds were observed for a limited period because of space limitations. However, in view of the fact that the

genital tract was definitely stimulated it would seem that a blood dyscrasia would have resulted if the hormone were going to have any effect on the hemopoietic tissue or peripheral blood.

The variation of the thrombocyte counts was considered to be in a normal range. Gray[5] and Dennington[4] have both reported wide ranges for thrombocyte counts. Similarly, Albritton[1] and Wintrobe[13] have reported a wide range in platelet counts in mammalian blood.

The experimental birds did not exhibit any clinical signs that could be associated with a hormone toxicity. They continued to eat and body weight of all birds increased daily. The lack of significant weight changes in gonads and adrenals indicated that the hormone as administered had no effect on these organs. No signs of inflammation were observed in the muscle tissue at the site of injection.

SUMMARY

The injection of a synthetic estrogen, estradiol cyclopentylproprionate (ECP), failed to produce any detectable clinical signs of toxicity in sixteen chickens. The hormone was used to study its effect on avian hemograms, blood clotting and hemopoietic activity.

1. Administration of the ECP in dosage as high as 12 mg (6 ml) failed to cause thrombocytopenia or produce hemorrhage.
2. The chickens receiving the hormone continued to appear clinically healthy.
3. Chickens receiving 0.1 percent sulfaquinoxaline in their ration did not show any different effects from ECP than those that were on a 22 percent protein broiler ration.
4. The gonads of the males and females of nine and one-half week old chickens did not show any effects from the administration of ECP but the mucosa of the oviduct and the albumin secreting glands that occur in the submucosa were stimulated.
5. Examination of the bone marrow at the time of necropsy did not reveal any gross changes in the marrow of ECP

injected chickens. Microscopic examination of marrow smears did not indicate an aplastic marrow was produced by the estrogen.

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THE TOXICITY OF CERTAIN ANTIBIOTICS AND FURAZOLIDONE FOR CHICKEN EMBRYOS

By R. F. GENTRY

Department of Veterinary Science
Pennsylvania State University
University Park, Pennsylvania

Received 14 July 1957

BEVERIDGE and Burnet[1] have described various techniques for the use of chicken embryos for the isolation and cultivation of viral and bacterial agents. Materials collected for virus isolation may contain various bacteria. In grossly contaminated samples, large amounts of certain antibiotics have been used to control bacterial growth. The amount of an antibiotic which could be safely tolerated by the embryos has not been established. When relatively large amounts of certain antibiotics were employed, an increase in the incidence of non-specific mortality was noted and the possibility of toxic reactions was considered.

It is reasonable to assume that there may be a wide range in the amounts of antibiotics and other bacteriostatic agents which may be injected into embryos without causing mortality. If toxic amounts were used, deaths would occur which, when combined with the deaths resulting from trauma, could make results difficult to interpret. In routine inoculations at this laboratory, mortality due to trauma has been relatively low, usually within the range of 3 to 5 percent. However, Bloom and Gordon[2] pointed out that although the

Authorized for publication on July 9, 1957, as paper No. 2166 in the journal series of the Pennsylvania Agricultural Experiment Station.

incidence of traumatic deaths from a single inoculation may be sufficiently low to be insignificant, when repeated injections were made into the same egg, the number of such deaths was great enough to interfere with the interpretation of the experiment. In studying this problem, they found that a satisfactory concentration within the egg could be achieved by introducing the test material into the air sac and allowing it to drop onto the inner layer of the shell membrane. It could then diffuse through the inner shell membrane where it was apparently taken up by the vascular system of the CA (chorioallantoic) membrane. They used such a technique to prevent excessive deaths due to trauma in testing the antiviral activity of various materials.

The purpose of this experiment was to determine the toxic amounts of certain antibiotics and furazolidone that would cause deaths in embryos when inoculated by different routes. When this is known, the amount of a given drug which will be tolerated by the embryos can be estimated and the problem of non-specific embryo mortality from this cause can be eliminated.

MATERIALS AND METHODS

Embryos. Ten-day old embryos were used for the inoculations. Although the toxicity of the drugs administered by different routes was to be compared, embryos of only one age were used. Inoculations into the yolk sac are most frequently made when the embryos are six to eight days of age, while ten-day old embryos are usually used for inoculations into the allantoic cavity. If different aged embryos were used for each of the different routes of inoculation, an accurate evaluation of the effect of the route of toxicity could not be made. Therefore, ten-day old embryos were used for all routes of inoculation. The choice of ten-day old embryos was based on the results of Smith and Thomas[5] who found that with the inoculation of bacterial endotoxins, ten-day old embryos gave maximal response while embryos younger or older than ten days gave little or no response.

Routes of inoculation. Three routes of inoculation were

employed, 1—AC (allantoic cavity), 2—YS (yolk sac), and 3—SM (inner shell membrane of the air cell). A small hole was drilled in the center of the air cell end of each egg. The AC and YS inoculations were made according to the technique described by Cunningham[3] with 1½ inch 24 gauge needles. The SM inoculations were carried out as described by Bloom and Gordon[2]. This consists of inserting the tip of the needle a short distance through the hole in the shell and dropping the material on the inner shell membrane. The needle must be placed in such a manner that the material will drop freely from the tip to prevent loss of a portion of the inoculum on the inner surface of the shell.

Inoculums used. The ten drugs listed in table 1 were employed. Sterile distilled water was added in amounts sufficient to give the desired concentrations and the volume containing a given amount of the drug was then inoculated. These volume ranged from 0.05 to 0.5 ml. This variation in volume did not affect the embryo mortality rate. Control inoculations with distilled water in amounts of 0.05 ml through 0.5 ml at increments of 0.05 ml were made by each of the three routes. Some deaths due to trauma occurred but the incidence was no greater in the groups receiving the large volumes than in those receiving the smallest volume.

Preliminary inoculations were made to determine the approximate toxic levels for each drug. These consisted of two or three widely different amounts which were inoculated into groups of at least ten eggs each. When the toxic level was not within the range selected, additional inoculations were made until the approximate toxic level could be estimated. Three or more closely spaced dosages near that level were then used in at least 10 eggs per dosage to determine the end-point. One inoculation containing a given amount of the drug being tested was made for each egg and all eggs candled at 24-hour intervals.

In preliminary tests, embryos were examined for lesions due to toxicity. The lesions observed included moderate to severe generalized edema and varying degrees of hemorrhage. The hemorrhages in the skin, usually consisted of numerous

TABLE I

The ELD_{50} of various materials inoculated by different routes

Inoculum	Route of Inoculation		
	SM*	AC Milligrams	YS
Achromycin HCl	0.96	3.2	5.2
Aureomycin HCl	1.5	12.0	6.5
Terramycin HCl	2.5	10.9	8.8
Chloromycetin	7.2	4.8	7.0
Neomycin	8.7	20.7	45.5
Bacitracin	9.9	30.0	50.0
Penicillin G. sodium	15.6	88.4	85.8
Dihydrostreptomycin sulfate	46.8	80.6	85.8
Furazolidone	15.0	4.2	3.0
Micronized furazolidone	18.0	2.0	0.7

* SM—innner shell membrane of the air cell
AC—allantoic cavity
YS—Yolk sac

petechia or small ecchymotic areas. The wings, legs, and head were most commonly involved but in some instances the entire embryo was affected. All embryos that died in the preliminary trials showed evidence of these lesions in varying degrees but none were found in embryos that survived more than 6 days following inoculation. Also, when the eggs were held in the incubator, no deaths occurred after the sixth day and the surviving embryos hatched at the same rate as non-inoculated eggs from the same source. It was, therefore, considered that examination of the embryos was not necessary in order to determine the end-point, and all subsequent determinations were based on embryo mortality. The variation in embryo susceptibility could not be controlled. This made it impossible to determine an exact end-point so the amount causing death in 50 percent of the embryos was calculated (ELD_{50}) using the method described by Reed and Muench[4].

RESULTS

The amount (milligrams) causing 50 percent mortality varied greatly with the individual drugs as well the route of inoculation. The most toxic of the antibiotics was achromycin hydrochloride which required less than one milligram

when inoculated on the SM (inner shell membrane of the air cell). The amounts then ranged up to 152.5 mg which was the ELD_{50} for dihydrostreptomycin sulfate when inoculated by the YS (yolk sac) route. The SM route was more sensitive than the AC or YS routes for all the antibiotics except chloromycetin. In this instance, the AC (allantoic cavity) route was most sensitive (4.8 mg) while the SM and YS routes were virtually the same at 7.2 and 7.0 mg respectively.

The results obtained with furazolidone and micronized furazolidone was almost identical but differed from the antibiotics in that the SM route was the least sensitive. The YS route was the most sensitive route for the furazolidones with the micronized material being the more toxic.

The ELD_{50} values for all the materials tested are shown in table 1. This shows the number of milligrams producing 50 percent mortality; therefore, the smaller the value, the more toxic the material.

DISCUSSION

From the results presented, it is apparent that the SM route was more sensitive than the AC or YS routes when the material being tested was soluble in water. The antibiotics were readily soluble at the concentrations employed and with the exception of chloromycetin, were able to diffuse through the inner shell membrane where they were apparently taken up by the vascular system of the CA membrane. After entering the blood stream they were carried directly to the embryo where they exerted their toxic action. The assumption that the antibiotic is taken up by the vascular system is substantiated by a comparison of the toxicity by the SM and AC routes. If the materials had merely diffused through the membranes into the allantoic fluid, the toxicity levels would have been approximately the same, presuming all the material reached the allantoic fluid.

Furazolidone is relatively insoluble in water (1 part in 40,000). When suspended in water and placed on the inner shell membrane, diffusion apparently does not take place. If the values obtained for furazolidone when given by the AC

and YS routes are compared to the results obtained for the antibiotics, it is apparent that the furazolidone is quite toxic for the embryos. The micronized furazolidone is merely furazolidone which has been ground to a very fine powder in an attempt to increase the solubility. The grinding apparently did not make the particles small enough to diffuse readily through the inner shell membrane since the toxic dosage was approximately the same as was determined for the regular furazolidone. The micronized material, however, was slightly more toxic than regular furazolidone when given by the AC and YS routes.

From the data presented, it is evident that the solubility of a drug influences its calculated toxicity when the SM route is used. A water soluble material diffuses through the inner shell membrane and is take up by the vascular system of the CA membrane. If a relatively insoluble substance is used, only a small portion of it penetrates the inner shell membrane. This would necessitate the use of a considerably larger amount to produce a toxic reaction by this route and the number of milligrams calculated as the ELD_{50} would be higher than it actually should be.

The smallest amount of any of the materials causing toxic deaths varied; but when one-half of the ELD_{50} dose was used, no deaths occurred. Therefore, one-half of the ELD_{50} dosage was considered to be the maximum amount that could be safely employed.

CONCLUSIONS

1. When 10-day-old chicken embryos were inoculated with eight antibiotics and two forms of furazolidone, deaths due to drug toxicity occurred during the first six days after inoculation with lesions of edema and hemorrhage. Embryos surviving after this time developed and hatched at a normal rate.
2. Antibiotics varied greatly in their toxicity, with the ELD_{50} of those tested ranging from 0.96 to 152.5 milligrams.
3. Toxicity was markedly influenced by the route of inoculation. Antibiotics were five to six times as toxic when

inoculated on the innner shell membrane of the air cell than when inoculated into the yolk sac or allantoic cavity.

4. Furazolidone, which is relatively insoluble in water, did not readily diffuse through the inner shell membrane and was least toxic when injected by this route.

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SURVIVAL OF NEWCASTLE DISEASE VIRUS IN NATURE

By ROBERT J. BOYD and ROBERT P. HANSON

Department of Veterinary Science and Bacteriology
University of Wisconsin, Madison, Wisconsin

Received 5 Aug 1957

THE transmission of Newcastle disease occurs through the elimination of infected exudates and excreta and the invasion of the digestive and respiratory tracts. The high stability of the virus of Newcastle disease makes possible

Paper NS *232* from the Department of Veterinary Science and Bacteriology, University of Wisconsin, Madison, published with the approval of the director of the Wisconsin Agricultural Experiment Station.

not only direct transmission but also indirect through contaminated soil and water (Brandly *et al.*[1])

Previous investigations have included studies of the effect of environmental temperature on viruses in soil (Olesiuk[3]) and water (Luginbuhl *et al.*[2]) The viability of a strain of virus in water was adversely affected by high temperature, chlorination, fluoridation, litter, feed, and fecal contamination, and by other non-specific factors but not by saline, rust or wide variation in pH (Winterfield and Seadale[6]). The survivial of Newcastle disease virus (NDV) in flat worms and annelids living in soil and water has not been investigated although the related virus of swine influenza was shown by Shope[5] to utilize the swine lungworm in a transmission cycle.

MATERIALS AND METHODS

The GB Texas strain of NDV isolated in May 1948 from an outbreak of nervous respiratory infection of chickens near Austin, Texas was used in all of the experiments. Filter paper, parr silt loam, lake water and distilled water were used as a supporting media for the virus. The paper discs which served as a control were 740-E of Schelicher and Schuell Co. Parr silt loam was obtained from the University Mandt farm and had a high water holding capacity, a large amount of organic matter with a high nitrogen content, and approximately a pH of 5. The pH of lake water was 7.6 and 8.1 before and after sterilization respectively. The pH of distilled water was 6.0 after sterilization. A species of earthworm of the genus *Helodrilus* was obtained in the vicinity of Madison, Wisconsin. Fresh water planaria were purchased from a commercial supply house and identified as *Planaria maculata* (*Dugesia tigrina*).

Paper discs and soil samples were seeded with allantoic-ammiotic fluids containing 10^{10} LD_{50} of Newcastle disease virus. The paper discs were 15 mm in diameter and absorbed 0.5 ml of the inoculum. The discs were held in an atmosphere of 0, 15 or 100 percent relative humidity maintained in glass chambers. Soil preparations of sterile and non-sterile soil, and of sterile soil seeded with bacteria, consisted of 2.5 g of

soil held in tubes. Earthworms were exposed to the virus by either placing them in infected allantoic-amniotic fluids or by inoculating each worm in the coelom with either 0.05 or 0.02 ml of NDV. Planaria were exposed by placing them in water containing the virus.

NDV was recovered from the paper discs by adding 4.5 ml of tryptone broth, macerating the discs with a sterile pipette, and using the supernatant as the source of inoculum. The same amount of tryptone broth was added to the 2.5 g aliquot of soil, the preparation shaken until the soil was distributed throughout the broth, the suspension centrifuged, and the supernatant used as the source of inoculum. A pool of three exposed earthworms or five exposed planaria were triturated in a TenBroeck grinder, the suspension centrifuged at 3000 rpm for 15 minutes, and the supernatants used as the inocula.

The antibiotics, streptomycin and penicillin, were added to the inocula to make a concentration of not more than 500 mg of the former and 500 international units of the latter for each 0.1 ml of the inoculum that was to be injected into each egg. Inoculation consisted of the injection with a 26 or 27 gauge needle of 0.1 ml of inoculum into the allantoic chamber of 10-day-old embryonating eggs. Six embryos were inoculated with each test material. NDV in the allantoic-amniotic fluids of embryos that died during the six days of observation was identified by the hemagglutination-inhibition test. The concentration of NDV in all inocula was determined by titrating its specific lethality in embryonated eggs. For a titration, 18 to 24 eggs were inoculated with three to four selected, successive, tenfold dilutions of the virus, each group of six eggs receiving one dilution.

RESULTS

The survival of NDV in paper discs held at 20 $\pm$2 C and subjected to three diverse relative humidities indicated that a high moisture content in the air was important for survival of the virus in the paper during the first 6 to 8 days. Paper discs held at 100 percent humidity remained

moist for the 20 day period; discs held at the lower relative humidities dried out within 24 hours. At the end of 3 weeks there was no significant difference in the amount of virus at 100 percent and zero percent relative humidity. Other studies have indicated that if observation had been continued beyond 3 weeks, virus would have persisted longest at zero percent relative humidity. When the log of the concentration of virus held at 100 percent relative humidity was plotted against time, a straight line was approximated (figure 1). This relationship was not obtained for the virus at lower relative humidities suggesting that not only were inactivating factors other than temperature involved when drying occurred but some protective factor also was introduced.

Soils maintained at all three humidities suffered a similar and small loss of virus during the first 8 days unlike the differential inactivation in paper discs (table 1). Between the 8th and 15th day, soil at 15 and zero percent relative humidity lost all virus activity. Soils held at 100 percent relative humidity had a $10^{3.3}$ LD_{50} titer of virus after 22 days. The soil at 100 percent relative humidity was moist at 22 days when the last recovery of virus was made: at zero percent humidity, the soil had lost most of its original moisture at 6 days.

To study the effect of bacteria in the soil on undiluted NDV, preparations of sterile soil, non-sterile soil, and sterile soil seeded with bacteria were used. The latter preparation was intended to compensate for the changes which might have taken place in soil which had been subjected to high temperatures of sterilization in the autoclave. No significant difference was found in the survival of undiluted NDV among the three soil preparations (table 2). The survival of diluted NDV with LD_{50} titers of 10^7 and $10,^5$ was studied in the three preparations of soil. Soils with a 10^5 and a 10^7 titer of NDV held at room temperature (20 $\pm$2 C) and 19 percent humidity retained viable virus for 19 hours and for 5 days respectively. A preparation having 10^5 LD_{50} of NDV held at 100 percent relative humidity and a slightly higher temperature of 25 C survived for 48 hours. A difference was not found

TABLE I.

Survival of Newcastle disease virus, 10^{10} LD_{50}, in paper discs and soils at 20 ±2C

Time (days)	100% Relative humidity			9-15% (Room) Relative humidity			0% Relative humidity		
	Paper discs	Soil Sterile	Soil Non-sterile	Paper discs	Soil Sterile	Soil Non-sterile	Paper discs	Soil Sterile	Soil Non-sterile
0*	10.0**	9.5	9.5	10.0	9.5	9.5	10.0	9.5	9.5
1	9.5	—***	—	7.8	—	—	6.6	—	—
2	—	—	—	—	—	—	6.2	—	—
3	8.3	—	—	6.5	—	—	>5.5	—	—
4	—	8.3	7.4	—	7.6	6.8	—	7.6	6.0
5	7.9	—	—	5.4	—	—	—	—	—
6	7.6	—	—	4.6	—	—	>6.5	—	—
8	—	7.8	8.3	—	7.3	7.5	—	7.4	6.4
9	6.2	—	—	>5.5	—	—	4.5	—	—
15	—	5.3	5.3	—	<2.0	<2.0	—	<2.0	<2.0
17	4.0	—	—	<2.6	—	—	4.6	—	—
20	2.8	—	—	2.8	—	—	3.0	—	—
22	—	3.3	3.3	—	—	—	—	—	—

*Titration at 1 hour
** Log LD_{50} of conc of virus
*** No recovery attempted.

TABLE II.

Survival of Newcastle disease virus, 10^{10} LD_{50}, in soil at 25 C and 100 percent relative humidity

Time	Sterile	Non-sterile	Sterile + bacteria
1 hour	9.5*	9.5	9.5
24 hours	8.6	8.2	8.5
72 hours	8.5 (8.0)**	7.4 (6.7)	7.6 (8.0)
5 days	6.5	6.5	7.4
6 days	5.6	5.6	6.0

* Log LD_{50} conc of virus
** Replicate

in survival of NDV among any of the dilutions of virus in the three soil preparations. Bacterial counts on the soil indicated larger numbers of organisms with an increase in the moisture content of the soil. Counts on the different non-sterile soil preparations ranged from 4.5 X 10^6 to 1.2 X 10^9. The pH of the soil preparations varied from 5.0 to 6.5.

The few observations on the survival of NDV in water indicated that aeration, pH, and the lack of salts and organic matter have an adverse effect on the viability of the virus. A preparation of NDV in sterile lake water held for 19 days at room temperature lost little infectivity dropping from $10^{8.0}$ LD_{50} to $10^{6.6}$ LD_{50}. Virus of the same initial titer was rapidly inactivated in aerated lake water; only $10^{0.8}$ LD_{50} could be recovered at 11 days. NDV in sterile distilled water was inactivated more rapidly than in lake water; at 6 days the titer had dropped from $10^{8.0}$ to $10^{6.5}$.

Virus in allantoic-amniotic fluids ($10^{9.0}$ LD_{50}) fed to earthworms held at 21 $\pm$2 C was inactivated between 3 and 4 days. The same titer of virus fed to earthworms maintained at 12 C remained viable for at least 18 days. A progressive but slower loss of virus was observed over a twenty-day period when either 0.05 or 0.02 ml of virus was inoculated into the coelom of earthworms held at 20 $\pm$2 C. When the loss of virus is plotted against the log of time, the notable fact is that the higher titer of virus inoculated, the greater the loss of virus (figure 2). The method of exposing fresh water planaria to NDV involved feeding a combination of liver

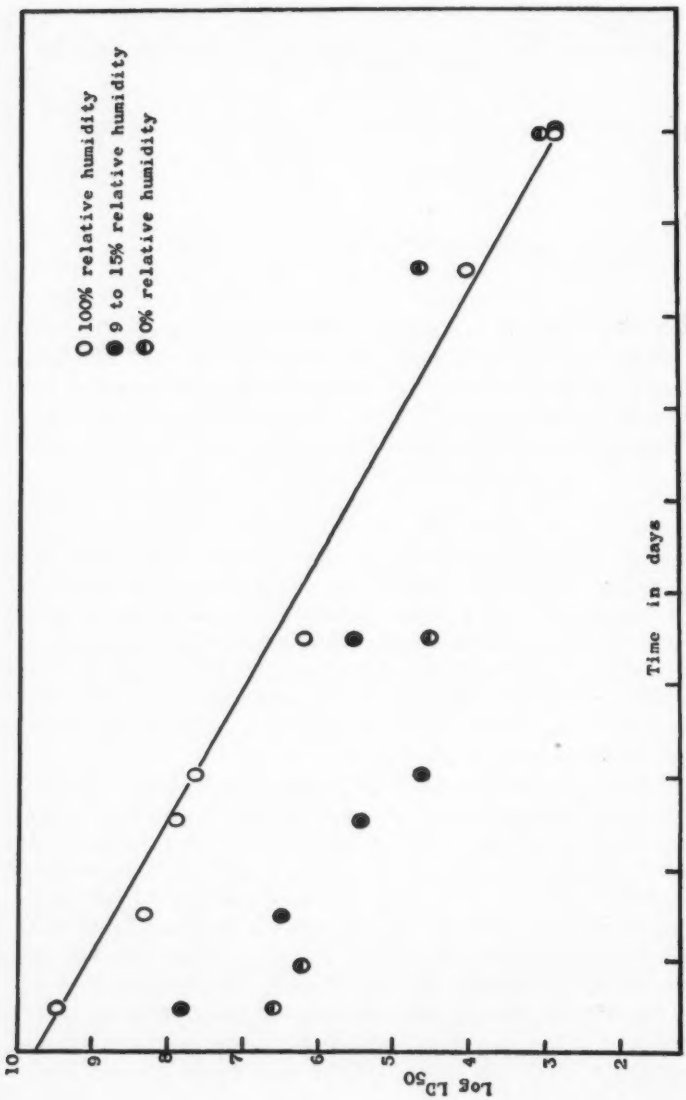

Fig. 1. The survival of NDV in paper discs at 20±2 C and at three relative humidities.

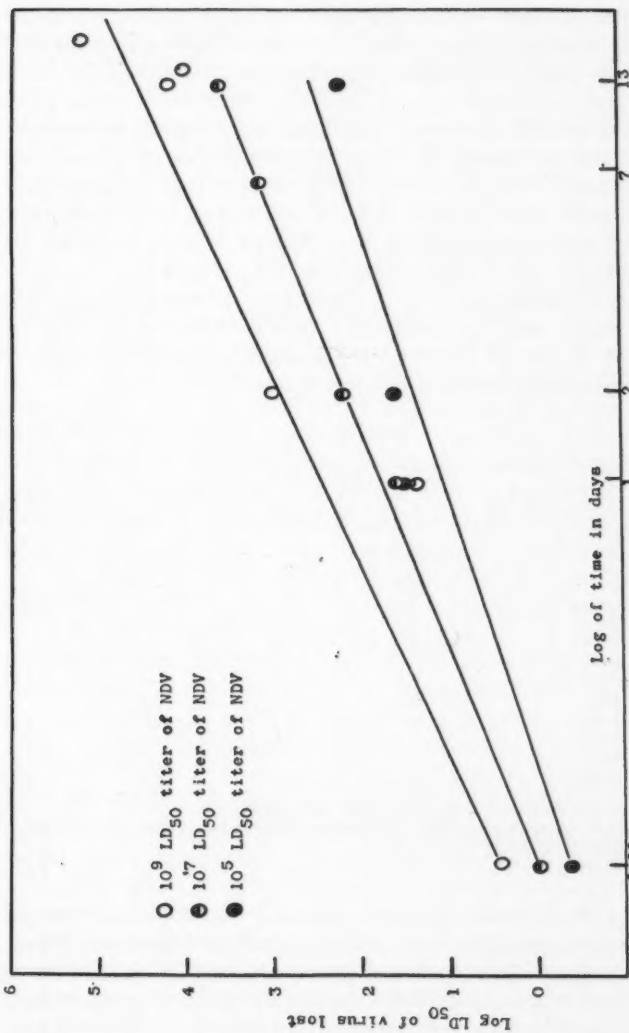

Fig. 2. The loss of Newcastle disease virus in earthworms inoculated with three LD^{50} titers of the virus (0.02 ml of virus in each earthworm).

and virus in lieu of feeding only virus. An experiment in which planaria were fed liver and virus, liver and virus followed by treatment with antiserum specific for NDV, and virus only indicated that virus was ingested rather than absorbed by the planaria, and that titers were more consistant when feeding planaria a combination of virus and liver. Planaria exposed to NDV were held at two temperatures, 20 ±2 and 31 C. The latter is near the maximum temperature at which the planaria can survive. The accumulative results of recoveries of NDV from planaria held at room temperature (20 ±2 C) indicated that there was no multiplication of the virus. NDV was demonstrated for as long as 9 days in planaria fed a 10^7 LD_{50} titer and held at 20 C and only 3 day in planaria held at 31 C (table 3).

TABLE III.

Survival of Newcastle disease virus in planaria at temperatures of 20 ±2 C and 31 C

Time planaria held after exposure to NDV*	Log LD_{50} titer obtained from 5 planaria	
	20 +2C	31 C
18 hours	6.3**	—***
24 hours	5.5	2.9, 2.6, <2.6
48 hours	3.6	3.4, 2.6, 3.3
3 days	4.5, 3.6	2.6, <2.6
4 days	—	2.6, <2.6
5 days	5.0	—
6 days	3.5	—
7 days	4.3	—
8 days	3.0	—
9 days	3.0	—
1 month	<2.6	—

* A 10^7 LD_{50} titer of NDV was fed to planaria
** The concentration of NDV per gram of planaria tissue (net weight).
*** No determinations were made.

DISCUSSION

The persistence of a virus which is eliminated by means of infected exudates and excreta, and which gains entry through digestive or respiratory routes may be furthered in the environment by protective substrates such as food, water, and soil, and invertebrates such as arthropods, flatworms and annelids.

A number of physical and chemical conditions acting together influence the stability of the virus whether it is in

soil or earthworms. As a basis of comparison with virus in soils and in animals where certain conditions could not be controlled, NDV in allantoic-amniotic fluids was added to paper discs which were held at 20 ±2 C and three selected relative humidities. The dry or wet state of the virus determined by the moisture present in the disc was important in regulating the rate of thermal inactivation. The inactivation NDV at 100 percent relative humidity in paper discs conformed to other studies at inactivation made on viruses (Pollard[4]). When the log of the concentration of virus was plotted against time, a straight line was obtained indicating a first order reaction. While a large quantity of the virus at the low relative humidities of zero and 9 to 15 percent was inactivated during the first 5 days, later, at 17 and 20 days, the quantity of virus recovered was equal to that obtained under 100 percent relative humidity. The virus at zero relative humidity was in the dry state, and if recoveries of virus had been attempted beyond 20 days, it is probable on the basis of other studies, that the virus would have survived longer in this dry state than in a state of hydration.

The rate of inactivation of the virus in soil was not observed to approximate the rate of inactivation in paper discs. In soil, virus persisted equally well at all three relative humidities, for the first 8 days, and became inactivated between 8 and 15 days at the lower humidities. The reason for the inactivation of NDV in soil between 8 and 15 days at the lower relative humidities while the control in paper discs survived for 20 days can only be postulated. Since approximately the same results were obtained with sterile and nonsterile soils, the possibility that bacteria or their products might have inactivated the virus was unlikely. Paper discs lost their moisture rather quickly as the surface was largely exposed. In contrast, the soil, the mass of which is less exposed, retained its moisture for a considerable length of time. It may be that the virus remained detectable in paper discs because the rapid drying process favored the conversion to an intact dehydrated virus structure, and in the soil the slow drying process failed to convert the virus to a viable, dehydrated form.

The importance of the transmission of NDV in water involves several factors of which the initial amount of virus in water, the dilution and dispersion of the virus during rains, the temperature, aeration, and pH of the water, and the accumulation of substances in the water either protective or adverse to the virus are a few. The few results obtained on the survival of virus emphasizes the adverse effects of aeration and pH, and the probable protective effect of salts and organic matter in water.

Little information exists on the relationship between helminths or annelids and viruses. The relatively short survival of NDV fed to earthworms compared to the prolonged survival of the virus inoculated into the coleom of the earthworm indicates that loss or inactivation of the virus was associated with the digestive processes of the earthworm. This was supported when a supernatant of earthworm tissue produced an inactivation that paralleled that in the coelom.

Planaria held at 20 ±2 C after being fed NDV retained the virus at least 3 times longer than planaria fed the virus and held at 31 C.

SUMMARY

The survival of (Newcastle disease virus) NDV in some of the substrates that might be utilized by the virus in natural transmission was investigated. These were sterile and non-sterile soils, water, earthworms, and planaria. Conditions which affected the survival of the virus in these agencies, such as relative humidity, pH, temperature, and bacteria were subject to control.

1. The survival of NDV in paper discs at 20 ±2 C and at three relative humidities served as a control of methodology.

2. NDV seeded in sterile and non-sterile soils maintained at 20 ±2 C and at 100 percent relative humidity survived for 22 days. Virus survived equally well for 8 days at zero, 15 and 100 percent relative humidity and considerable residual moisture remained in the soil. NDV in sterile and non-sterile soils at zero and 15 percent relative humidities was inactivated between 8 and 15 days when the soil became dry in contrast to virus held at those conditions in paper discs. The difference

in length of survival of virus in paper discs and in soil was possibly caused by the slow rate of change of the virus from the wet to the dry state in the soil preparations and the rapid change in paper preparations. The inactivation of the virus was not significantly different in sterile and non-sterile soils.

3. The survival of NDV in water was adversely affected by aeration and lack of salts and organic matter.

4. NDV fed to earthworms (*Helodrilus*) was inactivated more rapidly than virus inoculated into the coelom. NDV fed to planaria (*Planaria maculata*) held at 31 C was inactivated more rapidly than in planaria held at 20 ±2 C. The virus appears to be destroyed by the digestive or assimilative processes of the invertebrates.

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PHYSICAL-CHEMICAL TREATMENT OF INOCULA AS A MEANS OF SEPARATING AND IDENTIFYING AVIAN VIRUSES

By CARLOS ALFONZO QUIROZ* and ROBERT P. HANSON

Department of Veterinary Science and Bacteriology
University of Wisconsin, Madison, Wisconsin

Received 2 Aug 1957

PROCEDURES for the separation of a mixture of two or more viruses sometimes are needed to obtain a diagnosis and frequently are needed to establish the purity of a virus culture or vaccine. Three approaches have been used: the inoculation of hosts of diffrent susceptibilities, the inoculation of actively or passively immune hosts, or the inoculation of susceptible hosts with materials treated to selectively eliminate one or more viruses. This report is concerned with the latter procedure.

MATERIALS AND METHODS

Nine strains of five avian viruses were used. The B1 strain of Newcastle disease virus was originally isolated by Hitchner in Blacksburg, Virginia, the Roakin strain was isolated in Vineland, New Jersey by Beaudette, and the GB strain was isolated at College Station, Texas by Boney. These strains represent three pathogenic types of Newcastle disease virus and differ considerably in other known characteristics. Two strains of fowl pox were used, the Salsbury strain was a vaccine purchased on the market and HC, a strain isolated

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* Present address: Bloque 8 #2 La Barraca, Maracay, Venezuela.
Paper NS——— from the Department of Veterinary Science published with the approval of the director of the Wisconsin Agricultural Experiment Stations.

by Dr. Harr of Charles City, Iowa. Two strains of laryngotracheitis were used: ASL, a vaccine purchased on the market, and the HA strain isolated by Dr. Harr of Charles City, Iowa. One strain of bronchitis virus and one strain of the Crawley virus were used. The bronchitis virus was isolated by Elizabeth Upton, of Madison, Wisconsin, and the Crawley virus was isolated by Dr. Crawley of Guelph, Ontario.

All of the viruses were maintained by passage in embryonated eggs, either by inoculation into the allantoic chamber or inoculation on the chorioallantoic membrane. Allantoic and amnionic fluids from infected embryos served as the source of virus for Newcastle disease and bronchitis viruses. Amnionic-allantoic fluids and the chorioallantoic membranes were used as virus sources for the strains of fowl pox, laryngotracheitis and Crawley's virus. The harvested materials in all instances were centrifuged at 1500 rpm for ten minutes, the supernatant fluids decanted, sealed in 1 ml ampules and stored at —20 C until use. All of the strains of virus were lethal for embryos when given at 1-10, 1-100 and 1-1000 dilutions. Higher dilutions killed embryos in some instances and in others gave lesions without death.

The thermo-stability of each virus strain was determined by subjecting a sealed ampule of the virus to 56 $\pm$0.5 C in a water bath for 5, 15, 30, and 90 minutes. The pH stability of the virus strains was determined by the use of one percent sodium hydroxide and one percent hydrochloric acid. The first gave a final pH of 12 when combined with equal quantities of undiluted virus, and the latter gave a final pH of 2 when combined with equal quantities of the undiluted virus preparation. The virus preparations adjusted to a pH of 2 or pH of 12 were held for 60 minutes at room temperature and then inoculated in 0.1 ml quantities into 10-day-embryonated eggs, six eggs per preparation.

The stability of the viruses to six chemicals and one antibiotic was also determined. The chemicals, their concentration, the length of time which the virus was exposed to them and the temperature at which the exposure took place are reported in table I. In all instances an equal quantity of

TABLE I

Chemicals used for treating viruses

Material	Concentration	Exposure Time	Temperature (C)
Phenyl mercuric borate (Matheson)	0.01%	15 minutes	25
Phenyl mercuric borate (Matheson)	0.001%	15 minutes	25
Ethly ether (USP Merck)	20%	24 hours	5
Phenol (Merck)	1%	60 minutes	25
Cresol (liquor cresolus saponatus)	1%	60 minutes	25
Glycerin (in buffered saline)	50%	3 days	5
Glycerin (in buffered saline)	50%	4 days	37
Merthiolate (Eli Lilly)	0.02	60 minutes	25
Merthiolate (Eli Lilly)	0.025	60 minutes	25
Erythromcin lactobionate (Abbott)	0.5 mg	10 minutes	25

the virus preparation and the chemical at the concentration indicated were mixed, allowed to incubate and then inoculated into embryonated eggs.

At least two and sometimes three or four trials were made of each interaction. The specificity of the virus infection of each embryo was determined on the basis of the nature of the lesions produced and the length of the interval between inoculation and death of the embryo. The toxicity of the chemical was tested in all instances.

RESULTS

The treatments in group 1 table II resulted in the elimination of one or more viruses without the destruction of one or more viruses. Heating both strains of laryngotracheitis and both strains of fowl pox for 30 minutes destroyed all activity. The Crawley virus and one strain of Newcastle disease virus could be recovered even after an additional hour of exposure. A low pH destroyed both strains of laryngotracheitis and pox viruses, and did not destroy Crawley virus, bronchitis virus or two strains of Newcastle disease virus. One percent cresol destroyed all viruses except bronchitis and Crawley virus.

The treatment in group 2 destroyed one strain of one or more viruses but did not eliminate any single virus. Included here were one percent NaOH, 20 percent ethyl ether, one percent phenol and 50 percent glycerin. The materials in group 3 were without effect upon the viruses: 0.02 percent

TABLE II

Stability of five avian viruses to selected physico-chemical treatments as determined by the number of infections induced in six chicken embryos.

Treatment		Group 1								Group 2								Group 3						
		56C for min.				Cresol 1%		HC1 1%		NaoH 1%		Ethyl ether 20%		Phenol 1%		Glycerin 37 C		Merthiolate .02%		Erythromycin .5 mg		Phenyl mercuric borate .01%		None
		5	15	30	90																			
Aliquot		A	A	A	A	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	
virus and strain	NDV—B1	6	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6	6	6	6	6	6
	—Roakin	6	2	3	0	0	0	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	—GB	6	6	6	3	0	0	5	4	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	Bronchitis W	1	0	0	0	1	3	6	6	3	2	3	5	6	6			6	6	6	6	6	6	6
	Crawley—HB	6	6	6	6	6	5	5	2	6	6	5	3	6	6	6	6	6	6	6	6	6	6	6
	LT—ASL	1	0	0	0	0	0	0	0	3	2	4	5	6	6	0	0	6	6	6	6	6	6	6
	—HA	0	0	0	0	0	0	0	0	2	1	0	0	6	6	0	0	6	6	6	6	6	6	6
	Fowl pox—Sals	6	1	0	0	0	0	0	0	6	6	3	5	6	6	6	6	6	6	6	6	6	6	6
	—HC	6	0	0	0	0	0	0	0	0	0	3	4	6	6	6	6	6	6	6	6	6	6	6
	Control					0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Entry indicates number of specific infections in 6 exposures in one or two samples or aliquots.

merthiolate, 0.01 percent phenyl mercuric borate and 0.5 mg of erythromycin per ml. Higher concentrations of merthiolate were toxic for the embryo.

It is apparent that there is considerable strain variation in the resistance of avian viruses to inactivation by physical-chemical procedures. While such procedures should be studied with more strains of each of these viruses before generalization can be made, it appears that it should be possible by physical-chemical treatment of inocula to resolve mixtures of selected viruses. Such procedures would be particularly useful to separate fowl pox or laryngotracheitis from Crawley's agent since it is difficult, if not impossible, to eliminate these viruses by the use of specific immune serum. It would be premature to utilize physical-chemical criteria in the initial identification of avian viruses.

SUMMARY

1. Three strains of Newcastle disease virus, two strains of fowl pox, two strains of laryngotracheitis, one strain of bronchitis and one strain of Crawley's virus were subjected to physical-chemical treatment.

2. Heating to 56 C, exposure to a pH of 2 and to 1 percent cresol destroyed the represented strains of one or more viruses. Treatment with 1 percent NaoH, 20 percent ethyl ether, 1 percent phenol and 50 percent glycerin destroyed one or two strains of several viruses but did not distinguish any of the viruses.

3. Certain mixtures of avian viruses could be resolved by treating the inoculum.

EFFECTS OF *ASCARIDIA GALLI* ON CHICKS WITH INFECTIOUS BRONCHITIS

By W. MALCOLM REID
Poultry Department, University of Georgia, Athens, Ga.

DONALD D. PATE
Poultry Department, University of North Carolina, Raleigh, North Carolina

and

ALBERT L. KLECKNER
School of Veterinary Medicine, University of Georgia, Athens, Ga.

Received 12 June 1957

A HIGH incidence of light infections of the intestinal roundworm *Ascaridia galli* commonly occurs in broiler production. This is a remarkable build-up of worm population considering the fact that the minimum life cycle requires 5½ weeks and broilers are marketed at 9 weeks. Furthermore, the excellent balanced rations used for growing chicks has eliminated nutritional deficiencies which are important predisposing factors to heavier worm populations. A recently completed survey of broilers in north Georgia (Reid[11]), showed that 59 percent of 100 flocks had birds infected with roundworms at the time they were marketed. Each flock was represented by 10 bird samples. A total of 240 or 24 percent of the 1,000 birds examined was infected. The average number of worms in infected birds was 6.2. Two flocks could be classified as heavily parasitized with 100 percent infection averaging 33.2 worms each. The other 57 were classified as lightly infected with an average of 4.3 worms each.

* Journal Series Paper No. 45, College Experiment Station, University of Georgia, Athens, Georgia. This study was partially supported by a research grant from the Moorman Manufacturing Company of Quincy, Illinois.

Although the heavy infections probably caused serious losses, the economic significance of these light infections has not been studied sufficiently to make it possible to assess losses incurred from such parasitism. During the course of controlled experiments comparing weight gains, feed consumption, and feed conversion of parasitized and unparasitized birds, an accidental outbreak of infectious bronchitis occurred. Of the 90 parasitized birds, 5.5 percent died of the combined effects of parasitism and infectious bronchitis while none died in the 45 controls which had infectious bronchitis but no worms. It thus seemed desirable to study further the interrelationship between these two types of infection. Three additional experiments with induced bronchitis and single doses of *A. galli* are here reported.

REVIEW OF LITERATURE

Although much experimental work has been carried on with *A. galli,* no clear answer can be drawn from these experiments as to the effects of such parasitism on the birds. Ackert and Herrick[2] showed that heavy single egg dosages of 1,000 or more eggs produced marked inappetence, weight loss, and some deaths. Larger egg numbers caused higher mortality. Sadun[13] found death to occur between 6 and 19 days after infection with single egg dosages of 2,500 up to 72,000 eggs per bird. With lighter artificial infections Ackert and Wisseman[3] reported no statistically significant differences in weight gain, blood sugar and hemaglobin levels between controls and birds parasitized 2 and 4 weeks previously with 200 embryonated eggs. Their original data[14] shows some decrease in weight gain averages in the 15 parasitized birds 2 and 4 weeks after parasitizing.

Reidel[12] showed experimentally that birds parasitized with *A. galli* at 8 days of age and coccidia (*E. tenella*) at 36 days gained weight more slowly than control birds unparasitized with the nematode but parasitized with coccidia. Peterson and Kerr[9] have reported on the relationship between *A. galli* infection and natural Newcastle disease outbreaks or Newcastle disease vaccinated hatches.

Comparative data are limited but the authors suggest that the higher mortality which occurred in the New Hampshire vaccinated chicks than in White Leghorns may have been due to greater susceptibility of the New Hampshires to *A. galli*. This statement indicates the suspicion of the authors that *A. galli* was a contributing factor in higher mortality. Although "worms" have often been mentioned as a stress factor in consideration of "air-sac" and other disease conditions, there does not appear to be experimental work supporting this hypothesis. No references could be found on interrelationships between infectious bronchitis and worm infections.

MATERIALS AND METHODS

Broiler-type strains of White Rocks were secured day-old from nearby hatcheries. Sexed cockerels were used in the second and third experiments while in other experiments mixed sexes were used. Chicks were maintained in electrically heated batteries with wire floors and fed on commercial, non-medicated, starting ration. Chicks were vaccinated in the water with a B-1 strain of Newcastle disease vaccine during the first week. At three weeks of age the chicks were banded, weighed, and paired or matched in triplicate using a method similar to that of Gardiner and Wehr[5]. Experimental and control groups were then randomized in batteries and the birds individually weighed at weekly intervals. In experiments three and four, birds to be exposed to infectious bronchitis virus were isolated in another building from bronchitis-free birds. Individual weights were recorded at weekly intervals.

Ascaridia galli eggs were embryonated by a combination of techniques developed by Ackert[8] and by Hansen, Olson and Ackert[7]. Fungal and bacterial growth in the water suspensions of eggs was prevented by the use of one drop of 0.5 percent merthiolate in each Petri dish according to the methods of Hansen and Ackert[6]. Eggs were kept in water for 2 to 6 weeks with frequent aerations. For each experiment the same sugar solution mixture containing embryonated eggs from several worms was used on all parasitized birds. Eggs

were administered into the crop with a Stoll pipette giving 300 ± eggs per bird. The birds were sacrificed 3 or 6 weeks after parasitizing, and the entire intestine to the level of the cecum was flushed and, after putting the contents through a 100-mesh sieve, examined for parasites. It has been shown by Edgar, Davis and Frazier[4] that numerous small worms are found in the lower intestine and are missed if this part is not examined. Worms, together with a ruler scale, were photographed, the images projected and magnified on the screen a known number of times and were measured with an architect's wheel.

Diagnosis of infectious bronchitis was made on the basis of general symptoms followed by serum neutralization tests made with pooled blood serums from recovered birds 3 to 6 weeks after appearance of respiratory symptoms. With the accidental outbreak of infectious bronchitis in the first experiment the recovered birds showed a very high titer. Similarly birds exposed to infectious bronchitis showed a high level of immunity as measured by the serum neutralization tests whereas control birds not exposed showed no immunity. Infectious bronchitis virus was administered in the second, third, and fourth experiments by intratracheal inoculation of each bird with the "Gainesville" strain of the virus. This is known locally as a "hot" strain and is used to induce infectious bronchitis in pullets before they come into production.

RESULTS

Four different experiments were conducted which gave evidence on the harmful relationship between the viral infection with infectious bronchitis and the parasitic infection with the large intestinal roundworm. The first of these experiments involved a single dosage of *A. galli* eggs superimposed upon a natural outbreak of infectious bronchitis. The first signs of respiratory involvement were noted at 3 weeks of age on the day of the parasitizing.

The birds were matched in triplicate by weight and divided into three groups of 45 birds each. Each bird in two groups received 300 worm eggs while the third group was not para-

sitized and served as controls. The first parasitized group was killed and worms isolated three weeks after parasitizing and the second group after six weeks. During the first three weeks, two birds of the first group and three of the second parasitized group died. None of the controls died although the respiratory symptoms were equally prominent in these birds. Effects of infections on mortality are summarized in table I. The period of maximum effects of worm parasitism has been reported as 10 to 17 days by Ackert and Herrick[2] after receiving eggs. Infectious bronchitis likewise usually runs its course during three weeks, so measurement of the combined effects was concluded three weeks after infection. The absence of mortality in non-parasitized but bronchitis-infected birds suggested the need for further study of the effects of parasitism.

The second experiment was designed in a similar manner to the first except that infectious bronchitis virus was introduced by intratracheal inoculation. Each group contained 64 birds. Two birds from each of the two parasitized groups died during the first three weeks after infection while none of the controls died. This 3.2 percent mortality in birds with double infection was similar to that found in the previous experiment.

TABLE I

Effects of double or single infections of *Ascaridia galli* and infectious bronchitis on the mortality and weights of three-week-old broilers three weeks after infection.

Experiment No.	Treatment	No. birds	Percent mortality	Wt. in grams
1.	Bronchitis and worms	90	5.5	535
	Bronchitis without worms	45	0.0	539
2.	Bronchitis and worms	123	3.2	742
	Bronchitis without worms	64	0.0	725
3.	Bronchitis and worms	88	1.1	741
	Bronchitis without worms	44	0.0	756
	Worms without bronchitis	88	0.0	830
	No Worms or bronchitis	43	0.0	861
4.	Bronchitis and worms	50	2.0	602
	Bronchitis without worms	50	0.0	626
	Worms without bronchitis	50	0.0	694
	No worms or bronchitis	50	0.0	721

Since the first two experiments had no control birds not exposed to infectious bronchitis virus to compare with infectious bronchitis-infected birds, the third and fourth experiments included bronchitis-free birds. The 88 birds given worms and infectious bronchitis virus showed 1.1 percent mortality. Half of these birds were killed after 3 and half after 6 weeks. Forty-four birds were exposed only to the infectious bronchitis virus. Eighty-eight birds had worms without infectious bronchitis virus and were killed at 3 and 6 weeks respectively. Another group of 43 birds had neither worms nor infectious bronchitis virus. None of the birds outside of those with the double infection died in the 3 weeks period after infection. These results are in agreement on mortality with the previous experiments. A fourth experiment was conducted to test further the hypothesis that the combined infection would induce mortality. This time all groups of 50 birds each were killed and the experiment was concluded 3 weeks after parasitism. One of the birds with infectious bronchitis virus plus worm infection died (2.0 percent mortality) while none died in the single infections or the control groups. The evidence clearly indicates that the combined effects of parasitism and infectious bronchitis produced a detrimental condition. Mortality was induced in all four experiments while single infection of either worms or infectious bronchitis virus failed to cause mortality in any group in any experiment during the three week period following infection.

The effects of double infection on weight gain are also of interest. In all groups the birds averaged within one gram of each other on the day that they were given infections. In experiment 3, those with the double infection weighed 741 grams, those with bronchitis virus alone 756 grams, those with worms alone 830 grams and the non-infected controls 861 grams. Experiment 4 showed the same order of weights as experiment 3 at 3 weeks of age.

The weight gains in all four experiments were treated statistically. No significant differences between treatments

in weight gains were observed for experiments 1 and 2. In experiments three and four significant differences among treatments were noted (table II). The effects of bronchitis were highly significant while the effects of worms were significant. The birds without bronchitis gained faster than the infected birds, and the birds without worms gained faster than the birds with worms. There was no significant interaction between bronchitis and worms. The effects of worms alone on weight gain have been studied by using a combined probability statement on the four experiments here reported plus a fifth in which bronchitis did not occur. Worms appear to depress weight gains since the data showed significance at about a ninety percent level of probability. This is the same trend shown in the data of Ackert and Wisseman[3,15]. It may thus be concluded that this worm dosage causes slight weight depression and that likewise weight depression occurs more severely in birds with the double infection than those with infectious bronchitis alone. The latter would be more apparent had it not been necesasry to exclude the birds which died from double infection in the statistical study.

The effects of infection on feed consumption follow a similar pattern to that of weight gain. Accurate data for analysis was available only in experiment 4. During the three week period of observation birds with the double infection consumed an average of 77 grams of feed daily, with infectious bronchitis alone 86 grams, worms alone 98 and uninfected controls 100 grams. Observation of the data shows that groups with worms alone and worms plus infectious bronchitis virus showed the greatest decrease in feed consumption during the second week of parasitism. This corresponds to the period of maximum mucosal penetration of *A. galli* as reported by Ackert[1]. When daily feed consumption was treated by analysis of variance (table III), it is shown that infectious bronchitis virus induced a highly significant decrease in feed consumption during the three week period while worms in the two infected groups induced a significant decrease.

TABLE II

Analysis of variance comparing body weights at six weeks of age in experiments 3 and 4.

Experiment	Source	df	MS
3	Total	262	
	Groups	3	206,606**
	Error	259	6,047
4	Total	180	
	Groups	3	601,733**
	Sex	1	79,395**
	Groups x Sex	3	7,058
	Error	173	5,038

** Statistically highly significant

TABLE III

Analysis of variance for feed consumption in experiment 4.

Source	df	MS
Groups	3	2,355.15**
Total	83	
Days	20	1,499.90**
Error	60	120.58

Infectious bronchitis had no consistent effects upon worm length in comparing different experiments but it did reduce the worm number over bronchitis-free birds. In any one experiment, the difference was not statistically significant but it approached significance in all cases. In experiment 3, birds with double infection had 2.3 and 1.2 worms per bird 3 and 6 weeks after parasitizing as compared to 3.1 and 1.9 respectively in birds without infectious bronchitis virus killed at the same times. In experiment 4 the numbers were 3.2 with infectious bronchitis and 5.6 without the virus. When these data were combined into one probability statement the reduction of worm number due to bronchitis was found to be highly significant ($X^2 = 12.84$, 3 df.). The mechanism involved in this decrease in worm number is not clear. It has been shown that host starvation may result in worm loss with this species[10]. Since infectious bronchitis often results in inappetence, some of the worms may have been lost to host starvation.

The average number of worms in the various experiments here reported was 4.3 with a range from 1.2 to 9.9. This average figure includes birds killed 3 and 6 weeks after parasitizing with the 6 weeks number always lower than the 3 week groups. This drop would be expected due to continual losses. The average in these four experiments with induced infections is 4.3 worms per bird. This happens to be exactly the same number found in the lightly infected birds in the broiler survey. Thus, effects of parasitism have been properly duplicated in the experimental design.

DISCUSSION

The combined mortality in birds with the double infection in the four experiments here reported is 3.1 percent. This is a significant loss economically if it can be shown that such a loss is due to the double infection and not to chance. The fact that mortality occurred in the group with double infection in four different experiments but did not occur in any of the groups with single infection appears convincing but a statistical study should be made. A binomial test was used to test the hypothesis that probable mortality in the birds with single infection was equal to the probability of mortality with double infection. The best estimate of probability of mortality is $\bar{p} = \frac{x}{N}$. For the double infection $\bar{p} = .031$ while for single infection $\bar{p} = 0$. This difference is significant. The same hypothesis tested with individual experiments shows significance only with experiment 1.

Data here reviewed show some loss in weight and reduction in feed consumption in birds with the single infection of *Ascaridia galli*. Under these experimental conditions the weight loss occurred before the 6th week. It may be supposed that weight loss will occur in growing birds of any age about 2 weeks after ingestion of embryonated eggs. In broiler production, heaviest exposure to embryonated eggs is most likely to occur at around 5 to 9 weeks of age since the numbers of embryonated eggs do not build up on new litter until a few worms in the birds have reached sexual maturity. Broiler growers especially try to avoid stress factors during the

last weeks before marketing since that is the period when weight gains give the biggest economic returns. Possibility of retardation in weight gain plus the fact that roundworms may increase mortality in combination with infectious bronchitis and may depress weight gains in concomitant infections with coccidia (Riedel[12]) suggests that light worm infections may be economically deterimental in rearing young stock.

ACKNOWLEDGMENTS

The writers are indebted to James L. Carmon of the Department of Experimental Statistics, University of Georgia, for statistical studies made in this paper.

SUMMARY

1. A combination of infectious bronchitis and *Ascaridia galli* infection has resulted in a mortality of 5.5, 3.2, 1.1, and 2.0 percent with an overall average of 3.1 percent in four different experiments in 3-week-old chicks. None of the controls died with single infections of bronchitis or with worms alone.

2. Average weight gains were less with the combined infection as compared with single infections of bronchitis or with worm groups in two separate experiments. A worm infection of an average of 4.3 worms per bird uncomplicated by infectious bronchitis under experimental conditions produces some depression in weight gain. Combined data from five experiments showed significance at approximately the 90 percent level.

3. Feed consumption was depressed 22 percent over controls with double infection, 13 percent in infectious bronchitis and one percent in the worm infected groups in results recorded in one experiment. The decrease was statistically highly significant with infectious bronchitis and significant with worm infections.

4. These results indicate that worm infections by themselves or complicated with infectious bronchitis may sometimes result in economic losses in rearing young stock under commercial conditions.

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CASE REPORT—ASPERGILLUS MENINGO-ENCEPHALITIS IN TURKEYS AND DUCKS

By KLAUS HUBBEN

School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Received 21 June 1957

ASPERGILLOSIS in poultry, commonly known as "brooder pneumonia," usually affects the lungs and air sacs. This mycotic disease is seen most frequently in young birds. The source of the infection is often believed to be contaminated litter or feed, but at times its origin is not so obvious. Moist litter is particularly suitable for growth of molds. The usual symptoms include difficult respiration, gurgling and crackling noises in the thorax, and poor condition. The infection is especially severe in groups of young birds and there is commonly one or two per cent morbidity with a high mortality rate. After the birds attain full size and approach maturity the number of cases is usually negligible. Fungi of the genus *Aspergillus* are considered saprophytic when encountered contaminating surface lesions or in lesions of other infections. Nevertheless they are pathogenic in some instances[3].

Forgacs and Carll[6] have demonstrated toxic effects in chickens as well as other animals, fed the substrate from cultures of *Aspergillus*. At necropsy, these birds presented generalized congestion, hemorrhages in the skeletal muscles, gastrointestinal tract, and lungs. Thus, exposure to moldy feed may be doubly dangerous.

A review of the medical and veterinary literature reveals only eight articles about aspergillosis of the central nervous system in the last five years. The lesions reported in the medical literature[2,4,5,7,8,9,13] were granuloma, abscess, and

encephalitis. The symptoms and lesions we observed in turkeys were similar to Biester and Schwarte's description[1] as well as those reported by Raines[10]. Recently, three outbreaks in turkeys and ducks with lesions in the brain, came to our attention.

1. On April 12, 1956, the owner of a flock of 1200 Beltsville poults brought four live and several dead birds to the diagnostic laboratory*. They were 2½ weeks old. Nine birds had died and four live poults evidenced clinical symptoms of difficult breathing, twisting their heads over their backs (torticollis), walking backwards (retropulsion), and falling to the side or backwards (ataxia). The owner had observed and removed some lumps of moldy litter that the poults were pecking and scratching when they first exhibited symptoms.

At necropsy, one poult revealed button-like plaques on the air sac and an irregular, white area on the cerebellum which was approximately one-half centimeter in diameter. This lesion involved the meninges and extended into the brain (fig. 1). Cultures of the brain on Sabouraud's media and beef liver infusion media were negative.

Histologic sections were made of the cerebrum at the occipital level including areas of the thalamus with optic chiasma and the cerebellum with underlying medulla oblongata and optic quadrigemina. The cerebral section with basal ganglia revealed no pertinent lesions. Examination of the cerebellar sections stained wtih hematoxylin and eosin revealed a marked leptomeningitis. The cells in the meninges consisted predominantly of macrophages, heterophiles, and a few giant cells. The edges of the gross lesion previously described evidenced numerous heterophiles and lymphocytes which obliterated the normal histologic landmarks of the cerebellum. The central portion consisted of non-nuclear, necrotic material and scattered lymphocytes. Mycelia were present in this area (fig. 2). The surrounding brain tissue

* Poultry Diagnostic Laboratory, F. G. Sperling, Director, New Bolton Center, University of Pennsylvania, School of Veterinary Medicine, Kennett Square, Pennsylvania.

revealed numerous small vesicular nuclei identified as astrocytes (gliosis). In adition, there was marked hyperemia with endothelial proliferation and cuffing by mononuclear cells.

2. On March 11, 1957, the same laboratory received four 3-week-old poults. There were 5200 birds in the flock and losses had been two to three percent. The most prominent symptom was weakness of the legs. The litter at this farm was peanut shells. The owner had not observed any mold and

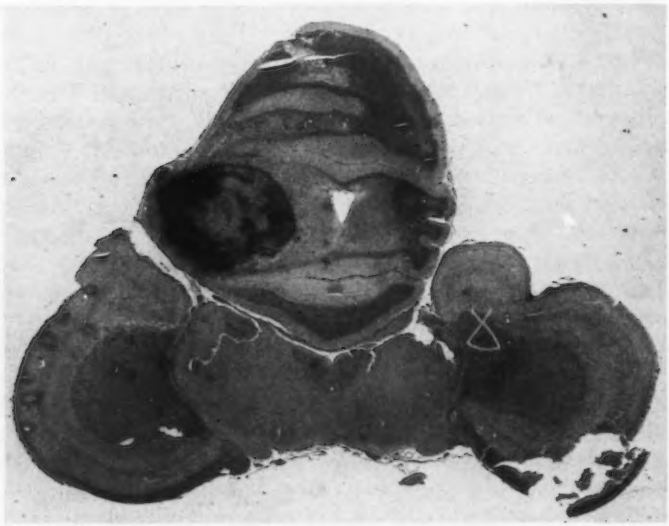

Fig. 1. Coronal section of the cerebellum. Note the destruction of the normal architecture with involvement of the meninges and displacement of portions of the surrounding tissue. Hematoxylin and eosin stain, 8×.

in this flock no additional cases have been observed to the present time.

Necropsy revealed the typical, button-like plaques of aspergillosis on the air sacs, pinhead-size spots in the liver, and a circular, white area below the surface of the left lobe of the cerebrum. This lesion was approximately two millimeters in diameter. A culture of the plaques on the air sacs and the brain lesion on Sabouraud's media was identified as *Aspergillus sp.*

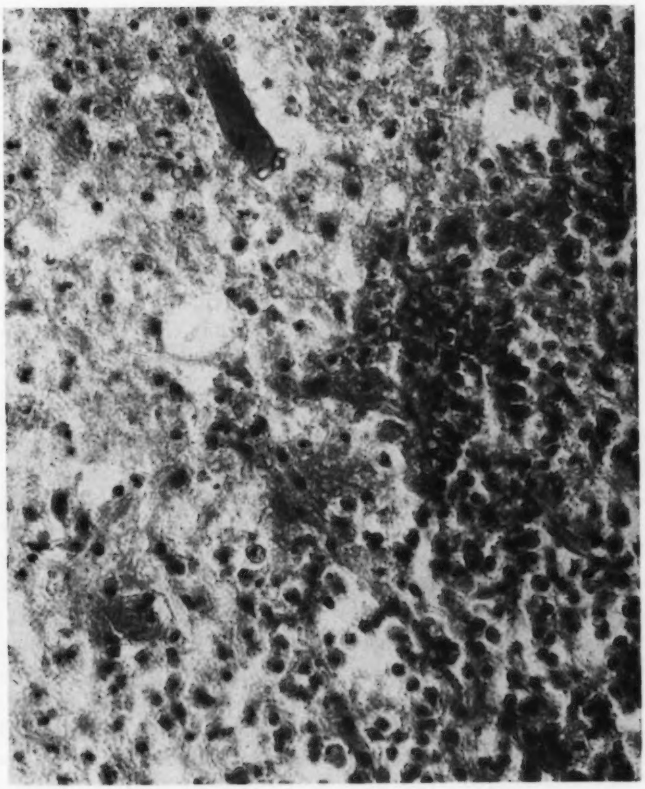

Fig. 2. Mycelia in an area of necrosis. Note the numerous lymphocytes. Hematoxylin and eosin stain, 950×.

The microscopic appearance of the brain lesions was similar to the first case, with disturbance of the normal structure, necrosis, and infiltration of the area by monocytes. The liver lesions were seen to be accumulations of monocytes and lymphocytes encountered in many conditions of domestic birds. No mycelia could be distinguished. A Gridley's stain of the liver was negative.

3. During the month of July, in 1953, ten Eider (*Somateria mollissima borealis*) ducklings died at the Zoological Garden in Philadelphia. Thirteen more died the next year, also in July. This represented approximately seventy-five percent of the total number of Eiders hatched in both instances. The ducklings were confined to a greenhouse which, in contrast to their natural North Arctic habitat, was extremely hot and humid. The symptoms of several included curling the head under the breast, loss of equilibrium, and lying on their backs with running motions of the legs.

At necropsy the lesions in the individual ducklings were almost identical. In the lungs and air sacs there were typical mycotic lesions. Eight of the ducklings revealed brain lesions, and with minor variations in size and location, those examined presented the following findings: the cerebral hemispheres revealed large, pink areas varying from two to eight millimeters in diameter. These were visible on the surface and extended into the brain. The livers of several also revealed a few white spots, one to two millimeters in diameter, located near the surface.

Microscopically the cerebrum revealed a large, necrotic area with numerous, engorged capillaries and arterioles in the central portion of the lesion. Around the periphery of the necrotic area, there was perivascular cuffing by monocytes, and gliosis. Some vessels were thrombosed. Groups of monocytes were seen clustered loosely around mycelia. The meninges were infiltrated by lymphocytes.

Examination of the lesions in the liver revealed necrotic areas but in this organ there was a more definite, granulomatous appearance. The necrotic centers with mycelia were surrounded by epithelioid cells and around the periphery were numerous lymphocytes, extending to surrounding areas to be enclosed in the hepatic sinusoids.

The ducklings hatched in 1955 were allowed to remain outdoors on a pond. Since then the acute infection has not developed, although occasional older birds have died of mycosis of the lungs and air sacs. No special susceptibility

among the several varieties kept on the pond was noted. The birds were emaciated and weakened to the point where other birds on the pond killed them. At necropsy there was frequently a widespread growth of mold in the air sacs which, upon closer examination, was classified as of secondary importance or terminal. However, since 1916, an average of one or two cases of chronic aspergillosis has been diagnosed each year. During this period the avian population at the zoo has varied from 800 to 1200. The lesions in these birds were encapsulated and resembled grossly a granulomatous disease. Chronic disease of this type, e.g., tuberculosis, actinomycosis, or certain neoplasms, is often associated with the finding of amyloid in the spleen, liver, kidney, and adrenals. The deaths of these semi-wild ducks appeared to be due to a related material seen in all the organs, most commonly in the spleen, kidney, pancreas, and liver. Large quantities of parenchyma were replaced by this substance. This material may be similar to hyalin or fibrinoid discussed by Selye: "Hyalin or fibrinoid is not a chemically well characterized material. It appears to result from the coacervation of the mucopolysaccharides of the ground substance with an alkaline protein. It is not chemically identical in every instance allegedly because the precipitant varies or additional substances may co-precipitate. Its close relationship to blood-globulins and amyloid have been emphasized, but a proper chemical designation of the substance would be impossible on the basis of available data"[12]. The substance encountered in older ducks was not true amyloid since it lacked the typical chemical properties, e.g., reaction with iodine. Nevertheless, it resembled amyloid in appearance and the circumstances under which it was found. It undoubtedly interfered with the function of these organs, contributing to the death of the birds[11].

SUMMARY

Three outbreaks of aspergillosis of turkeys and ducks with lesions in the brain and meninges are described.

The symptoms as well as the macroscopic and microscopic appearances of the lesions suggest a more acute disease process than is usually attributed to organisms of the genus *Aspergillus*.

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RESEARCH NOTE—THE INEFFECTIVENESS OF FURAZOLIDONE AS A PROPHYLACTIC AGENT AGAINST TURKEY ORNITHOSIS

By D. E. DAVIS, J. R. WATKINS, and J. P. DELAPLANE
Texas Agricultural Experiment Station, College Station, Texas

Received 1 Aug 1957

IT IS recognized that furazolidone has been effective in the treatment and prevention of several bacterial diseases.[1,2] Its efficacy against turkey ornithosis virus was not known and these experiments were designed to test its effectiveness.

MATERIALS AND METHODS

In this study, two similar experiments were conducted at different times. In the first experiment, 125 3-week-old broad breasted bronze poults were divided into five equal groups. Group 1 served as inoculated controls. Groups 2, 3, and 4 were inoculated with ornithosis virus and immediately given an all-mash feed containing 100, 200, and 300 gm furazolidone per ton, respectively. Treatment was administered for 2 weeks. Each turkey was inoculated intratracheally with 0.25 ml of amnio-allantoic fluid. The inoculum was egg propagated, fifth passage Jo strain ornithosis virus. Group 5 served as uninoculated, untreated, isolated controls.

All birds which died were necropsied. Virus isolation attempts were conducted on representatives from each group. All inoculated survivors were killed and assayed individually for virus. Virus isolation attempts were conducted in 7-day-old chicken embryos.

Furazolidone (NF-180) supplied by Hess and Clark, Inc., Ashland, Ohio.

TABLE I

The daily mortality of 3-week-old poults infected with ornithosis virus and treated with various levels of furazolidone.

Group number	Number poults	Furazolidone gm per ton	Mortality by days*											Total mortality	Virus recovery
			4	5	6	7	8	9	10	11	12	13	14		
Experiment 1															
1	25	0	0	0	1	3	0	3	3	4	4	3	2	23	100%
2	25	100	1	0	0	1	1	1	2	3	2	5	1	17	100%
3	25	200	1	0	0	4	2	3	3	2	1	1	3	20	100%
4	25	300	0	0	0	3	4	5	2	1	4	0	1	20	100%
5	25	0	0	0	0	0	0	0	0	0	0	0	0	0	—
Experiment 2															
1	20	0	1	1	7	4	3	3	1	—	—	—	—	20	100%
2	20	100	0	2	6	7	0	2	1	1	0	0	0	19	100%
3	20	200	0	2	4	7	5	1	1	—	—	—	—	20	100%
4	20	300	0	0	10	3	3	2	0	1	1	—	—	20	100%
5	20	0	0	0	0	0	0	0	0	0	0	0	0	0	—

* No mortality occurred during days 1, 2, and 3.

In the second experiment, 20 poults were used in each of the five groups.

RESULTS

Mortality of the poults in experiment 1 and 2 are shown in table I. Ornithosis virus was recovered from representative dead poults and from each survivor in group 1 through 4 in both experiments. All inoculated birds showed symptoms and lesions similar to those previously reported for ornithosis in turkeys.[3]

SUMMARY

Furazolidone had no prophylactic value against turkey ornithosis under the conditions of this experiment.

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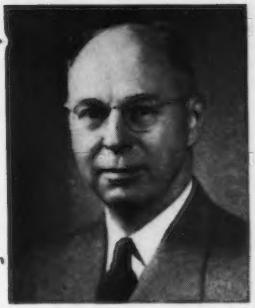

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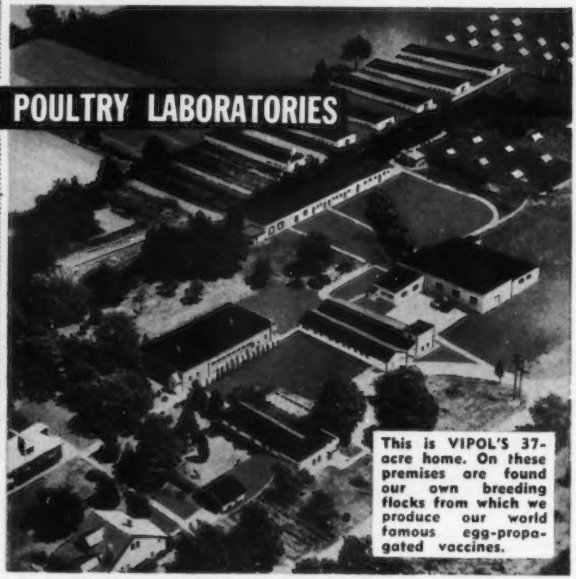

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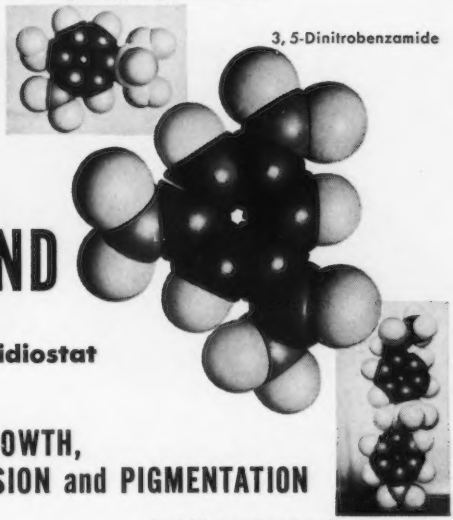

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